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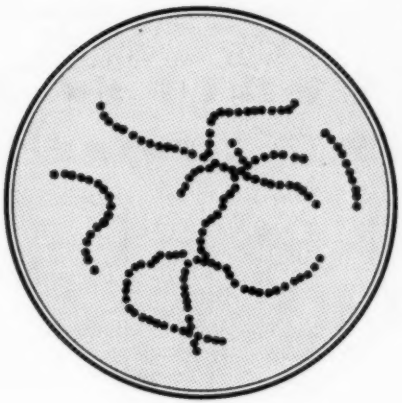

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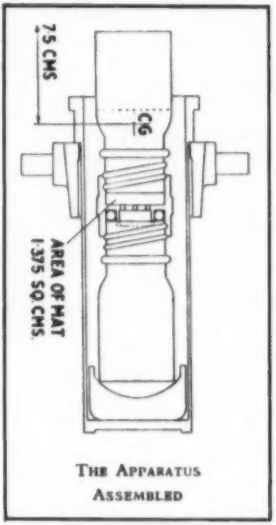

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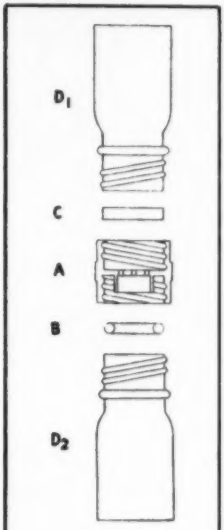

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One Manufacturer's Opinion

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Observing the success of quality control in industry, professional groups are asking why quality control techniques should not be applied to other areas. Recent events point up demands by leading scientists for this application—especially to those areas dealing with the safeguarding of human life.

Aside from the particular product or service offered by a manufacturer, long-term success is, after all, dependent on the guarantee of reliability and quality offered the consumer. Knowing this, industry has developed rigid quality control over production. Extensive testing, taking weeks or months, is undertaken before release of a product run. This testing serves a dual purpose. First, efficiency in manufacturing is maintained and guarded; secondly, the product or service offered is kept at a standard to satisfy the discriminating public.

In the clinical laboratory, advances made in specific instrumentation, methods, technique, and diagnostic reagents have, until recent years, greatly exceeded the overall quality control practiced over biochemical determinations.

It is not surprising that modern advances and antiquated control are functioning side by side in laboratory testing. *Adequate control measures have not been available.*

Now, two major steps have been taken to fill this void. First, the application of statistical measures of precision and of accuracy, and the routine use of the specific deviation yardstick are becoming widespread *in revealing* laboratory efficiency problems. Secondly, the common usage of two important standards-in-serum for biochemical testing, Versatol (normal) and Versatol-A (abnormal), with known absolute values in the normal and abnormal range, meet the demands of advanced laboratory analyses.

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Clinical Pathologists, Chemists Eye Application of Industrial Advances to Laboratory

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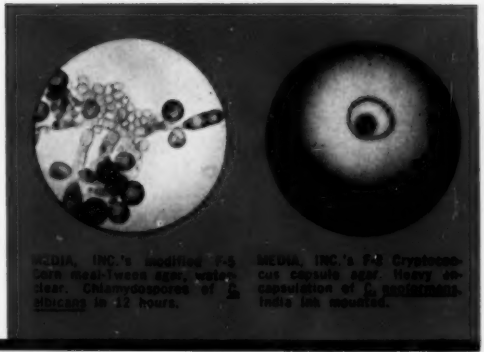

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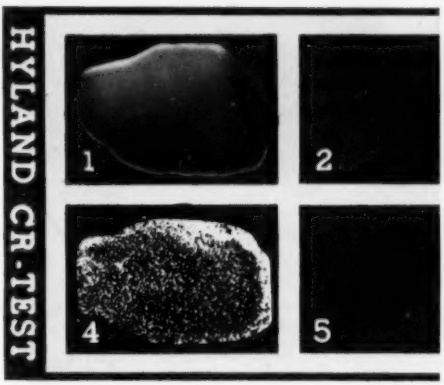

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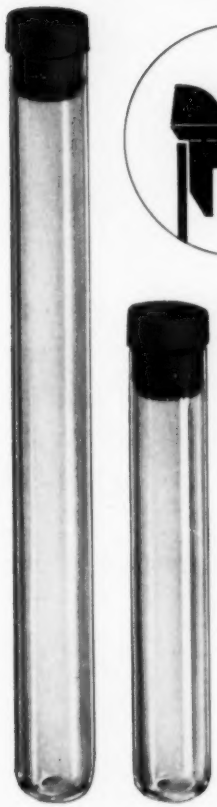

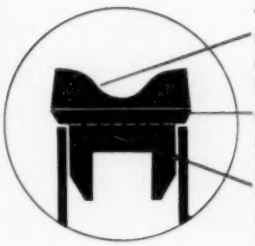

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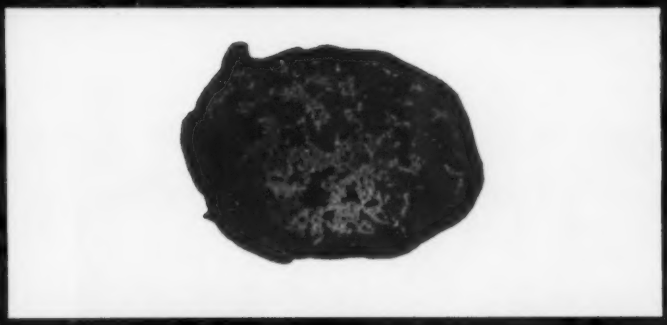

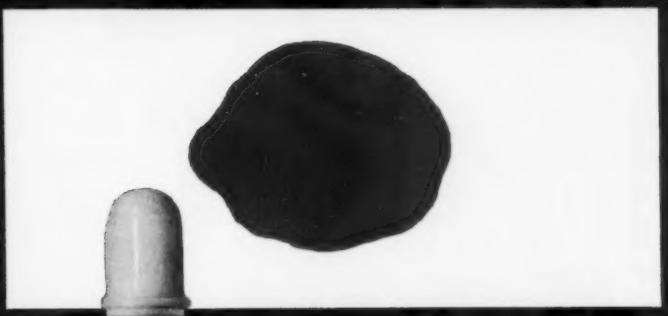

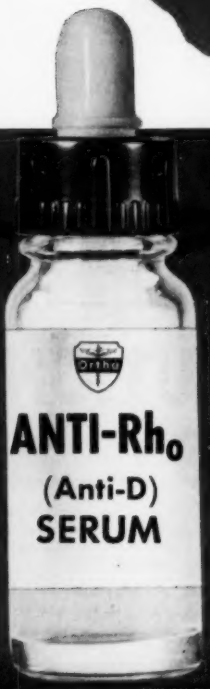

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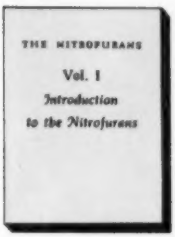

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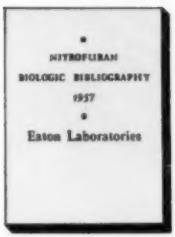

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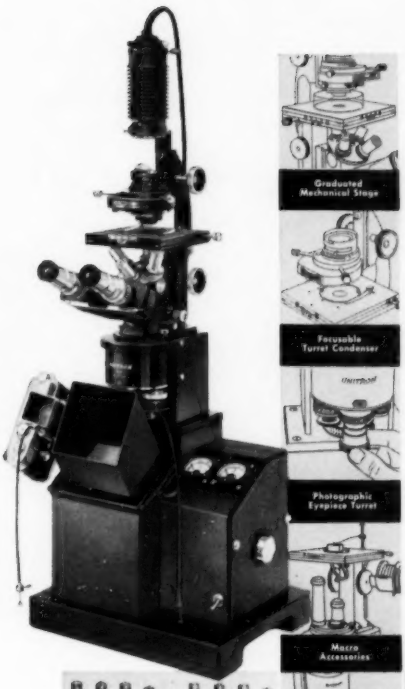

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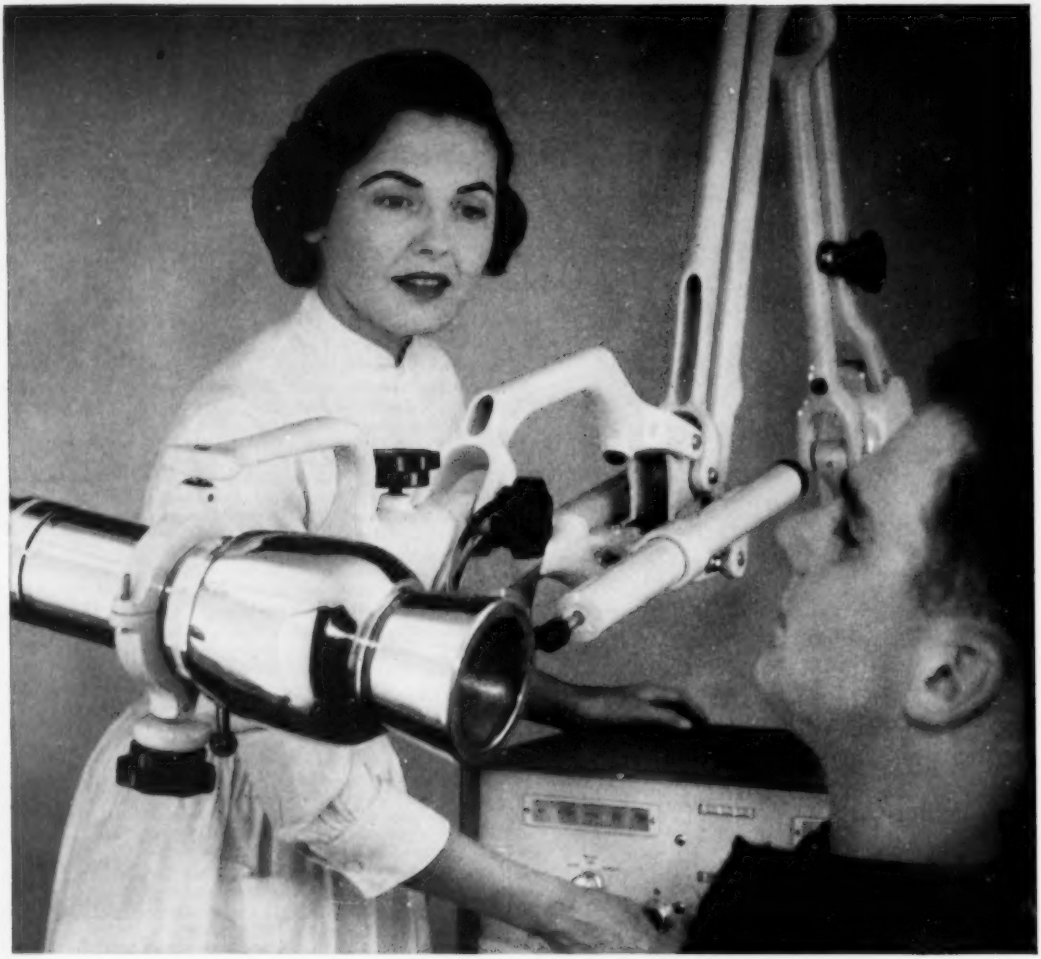

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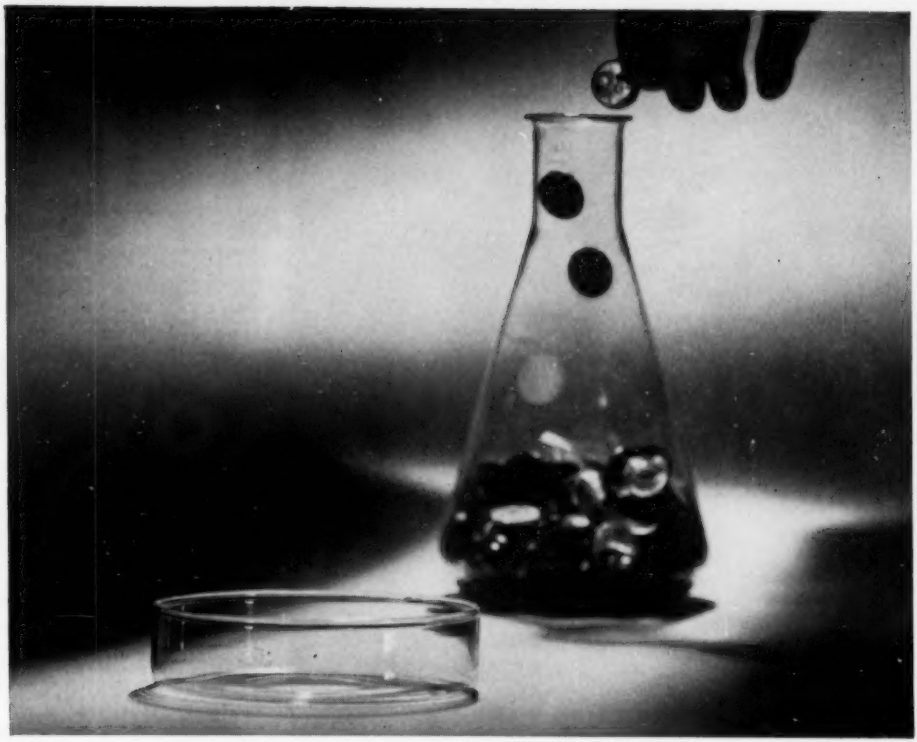

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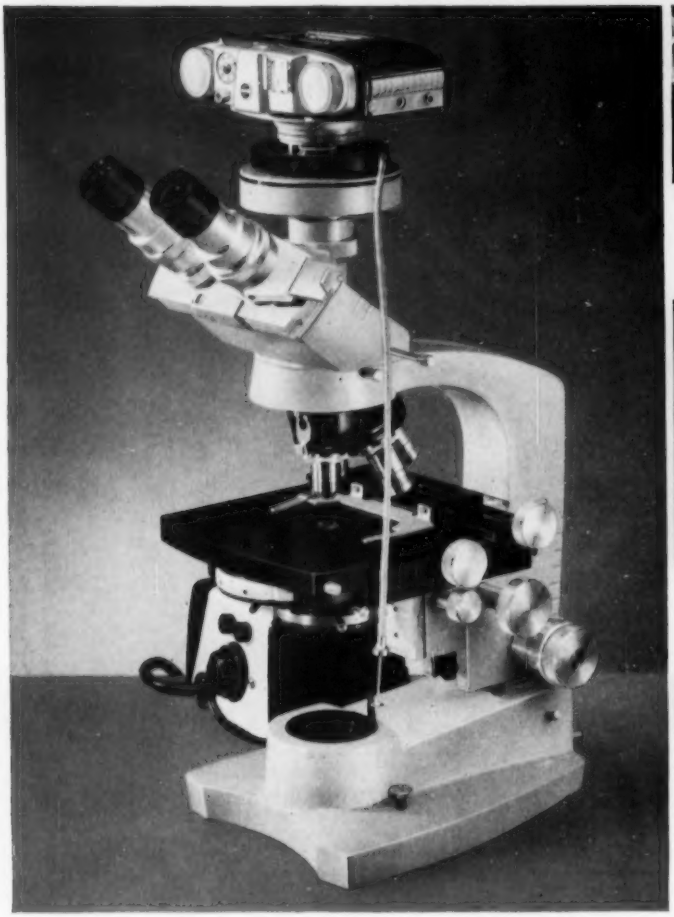

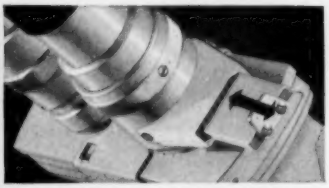

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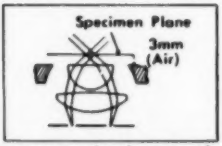

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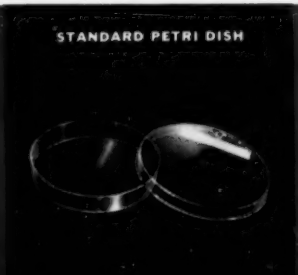

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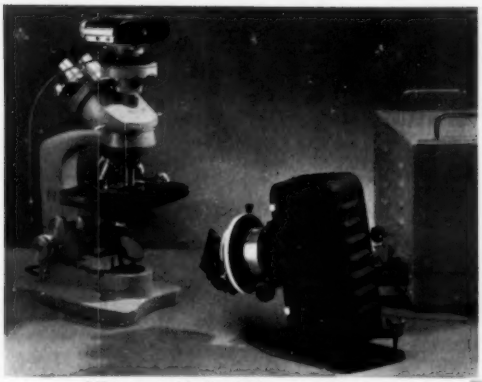

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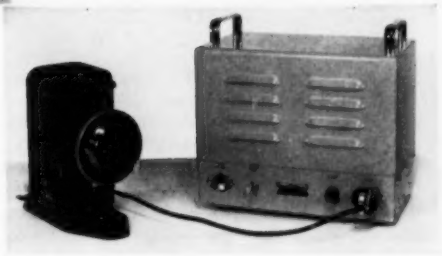

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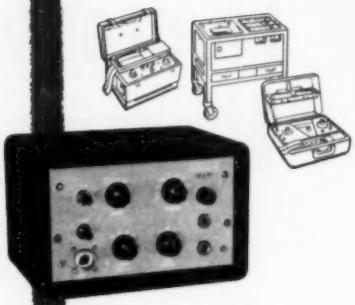

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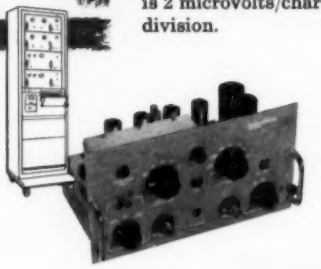

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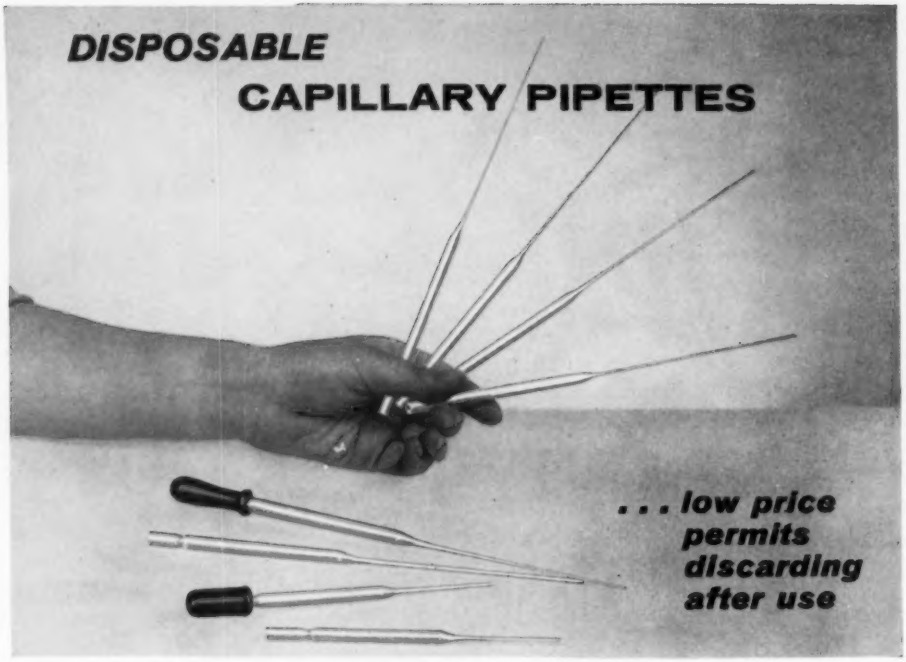

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CLINICAL AND EXPERIMENTAL

STUDIES ON BLOOD LIPIDS DURING EXERCISE

I. ARTERIAL AND VENOUS PLASMA CONCENTRATIONS OF UNESTERIFIED FATTY ACIDS

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STOCKHOLM, SWEDEN

THE quantitative interrelationship between the oxidation of carbohydrates and lipids in the muscular tissues during exercise is little understood. Studies of the respiratory quotient during moderate exercise generally have shown it to be about the same as at rest (e.g., about 0.85) indicating that various kinds of foodstuffs are burned.[1] Studies in human beings have shown that neither in the beating heart[2] nor in the resting forearm[3] is the extraction of carbohydrates enough to account for the total oxygen consumption. Bing and associates further demonstrated[4] that the heart extracted a great deal of fatty acids which, if completely burned, could account for about 70 per cent of the oxygen consumption. In a recent paper Gordon gave evidence that the principal type of lipids being extracted by the heart are the unesterified fatty acids, UFA.[5]

The presence of UFA in human plasma has been known for about 100 years.[6] The greatest part is associated with albumin.[7-9] This soluble complex rapidly exchanges with the extravascular compartments.[10, 11] During 1956, work appeared indicating that UFA were a metabolically highly active component.[5, 12, 13] The different findings were interpreted to show that UFA represented the blood lipid fraction which primarily was utilized and ready for metabolization in the tissues. More substantial evidence for this was obtained

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when McCalla and co-workers[14] demonstrated the rapid appearance of $C^{14}O_2$ in the expired air of fasting rats after administration of albumin-bound palmitate–1—C^{14}.

It was considered of interest to study the effect of muscular work on UFA in man. In the present study the extraction of unesterified fatty acids by the exercising leg has been investigated.

MATERIALS AND METHODS

Healthy volunteers between 20 and 35 years of age were studied. The experiments were performed in the morning 1 to 2 hours after a light breakfast, except in two of the subjects who had fasted for 36 hours.

After local anesthesia, polyethylene catheters were inserted percutaneously in one brachial artery and in both femoral veins. The femoral veins were punctured about 2 cm. below the groin and the catheters inserted 5 to 10 cm. distally. The exercise was performed on a bicycle ergometer usually in the sitting position, sometimes in the supine position. The work was performed exercising one leg at a time, the resting leg being put in a position as convenient as possible. The ergometer was supplied with a specially arranged spring which made the return of the pedal passive. The exercise consisted of an uninterrupted series of work starting with a load of 150 to 300 Kg. per minute and successively increasing every 4 to 6 minutes by a further 150 to 300 Kg. per minute. The exercise was performed until the subject had performed his utmost and further exercise was impossible due to exhaustion of the exercising leg. Blood was withdrawn from the 3 different sources simultaneously into silicone-treated, heparinized syringes. It should be mentioned that no heparin was introduced into the blood stream during the experiment.

Lactic acid was determined according to Barker and Summerson[15] as modified by Ström.[16]

For estimation of UFA the blood was immediately centrifuged, and the plasma pipetted into chloroform-methanol within 5 minutes after withdrawing. The lipids were extracted with chloroform-methanol and the extract treated as described earlier[17, 18] with the exception that the extract was washed with an acid phosphate buffer to prevent the movement of any UFA into the methanol-water phase.[19] After separation of the phospholipids on silicic acid, aliquots were taken of the chloroform phase, evaporated, dissolved in ethanol, and titrated for fatty acids with 0.02 normal aqueous sodium hydroxide delivered from an Agla microburette. Thymol blue was used as indicator. Nitrogen gas was bubbled through the solution all the time during titration. Blanks and standards of palmitic acid were run simultaneously. The standard error for a single determination was 0.02 mEq. per liter. All samples were titrated in triplicate. With this technique only the long chain fatty acids are estimated.

RESULTS

The arterial level of unesterified fatty acids decreased or remained fairly constant during exercise (Fig. 1). During a resting period of 30 minutes after the exercise the UFA level increased and decreased again in the two subjects who repeated the exercise with the other leg.

The arteriovenous difference data can be divided into 3 main groups, i.e., resting levels of UFA, UFA levels at moderate muscular exercise, and levels at maximal exercise. The first two groups have been listed in Table I.

The resting levels showed a negative arteriovenous difference except in one of the two fasting subjects. This difference varied between –0.03 and –0.23 mEq. per liter. After 4 to 6 minutes at different moderate work loads this difference had shifted in the working leg, the venous concentration being lower than the arterial. The mean value for this difference was 0.11 mEq. per liter.

Also in the resting leg there was a similar tendency in more than half of the tabulated work loads, although to a lesser degree than in the exercising leg. The mean value for the arteriovenous difference in the resting leg was 0.02 mEq. per liter. No correlation could be observed between the work load and the arteriovenous UFA concentration difference in either leg.

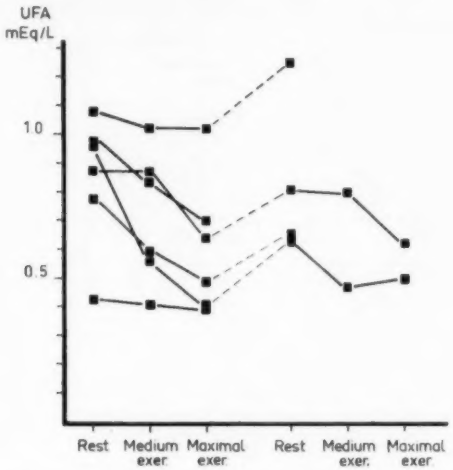

Fig. 1.—Arterial concentration of unesterified fatty acid in 6 subjects during exercise (——) and a resting period of 30 minutes (- - - -).

At maximal exercise, on the other hand, the venous concentration of UFA in the exercising as well as in the resting leg was in all cases but one of the fasting subjects equal to or higher than the arterial UFA level (Table II).

The relation between arterial and venous UFA concentration and, on the other hand, the lactic acid concentration during exercise with one leg can be seen in Fig. 2. That UFA were extracted from the blood perfusing the exercising leg at the different work loads up to the maximal work is seen from the differences in concentration of the arterial and venous blood. At 750 Kg. per minute with a pulse frequency of 165 beats per minute, however, the UFA levels at the different sites become equal. At this work load the lactic acid shows a considerable increase, increasing from about 3 mEq. per liter at 600 Kg. per minute to about 5 mEq. per liter. Furthermore, the considerably higher venous lactic acid concentration in the exercising leg as compared to the resting leg can be seen.

In Fig. 3 an experiment is recorded where the subject worked first with one leg and then, after resting for half an hour, he worked with the other leg. Principally the same events can be seen as in Fig. 1. The initially negative arteriovenous UFA difference shifts during the two first work loads to become positive. At maximal work with venous lactic acid concentrations above 5 mEq. per liter the arteriovenous UFA difference returns toward a negative

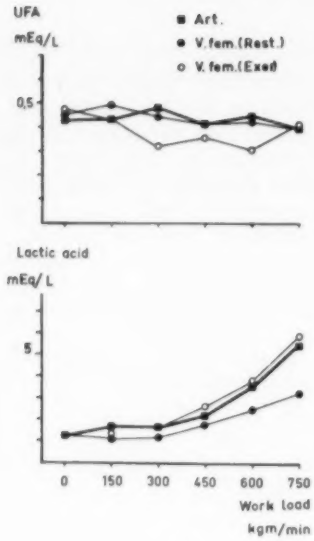

Fig. 2.

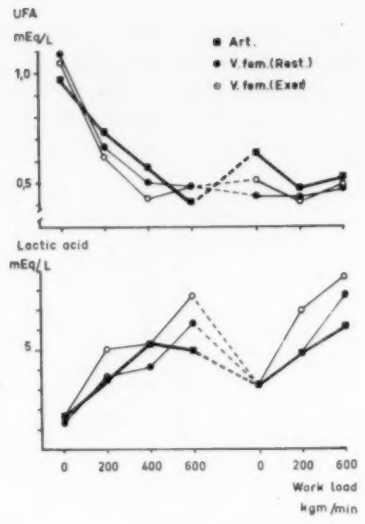

Fig. 3.

Fig. 2.—Arterial and venous concentrations of unesterified fatty acid and lactic acid during one leg exercise.

Fig. 3.—Arterial and venous concentrations of unesterified fatty acid and lactic acid during exercise first with one leg and then with the other after resting for 30 minutes.

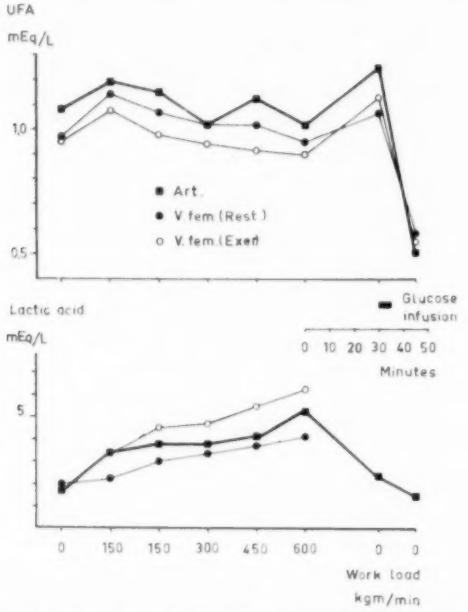

Fig. 4.—Arterial and venous concentrations of unesterified fatty acid and lactic acid during exercise with one leg. The subject had fasted for 36 hours. The first sample at 150 Kg. per minute was withdrawn after 1 minute. After resting for 30 minutes, 12.5 Gm. of glucose was infused.

value. It is also of interest to note that at rest while the lactic acid concentration decreases the arteriovenous differences of unesterified fatty acids again is reversed, indicating an extraction of the fatty acids also during the resting period.

TABLE I. ARTERIAL AND VENOUS PLASMA CONCENTRATIONS OF UFA AT REST AND AT MODERATE EXERCISE WITH ONE LEG

CASE NO.	WORK LOAD (KG./MIN.)	UNESTERIFIED FATTY ACID CONCENTRATION (MEQ./L.)			ARTERIOVENOUS DIFFERENCE (MEQ./L.)	
			VENOUS			
		ARTERIAL	RESTING LEG	EXERCISING LEG	RESTING LEG	EXERCISING LEG
1	0	0.98	1.13	1.07	−0.15	−0.09
	300	0.83	0.88	0.77	−0.05	0.06
2	0	0.97	1.09	1.05	−0.12	−0.08
	200	0.73	0.66	0.62	0.07	0.11
	400	0.56	0.48	0.42	0.08	0.14
3	0	0.87	1.00	1.10	−0.13	−0.23
	300	0.87	0.82	0.75	0.05	0.12
4	0	0.43	0.46	0.47	−0.03	−0.04
	150	0.43	0.48	0.43	−0.05	0.00
	300	0.48	0.44	0.32	0.04	0.16
	450	0.41	0.41	0.35	0.00	0.06
	600	0.44	0.42	0.30	0.02	0.14
5*	0	1.08	0.97	0.95	0.11	0.13
	150	1.15	1.07	0.98	0.08	0.17
	300	1.02	1.02	0.94	0.00	0.08
	450	1.12	1.02	0.92	0.10	0.20
6*	0	0.78	0.92	0.85	−0.14	−0.07
	150	0.69	0.66	0.58	0.03	0.11
	300	0.59	0.68	0.52	−0.09	0.07
	450	0.57	0.60	0.50	−0.03	0.07

*Fasted for 36 hours.

TABLE II. ARTERIAL AND VENOUS PLASMA CONCENTRATIONS OF UFA AT MAXIMAL EXERCISE WITH ONE LEG

CASE NO.	UNESTERIFIED FATTY ACID CONCENTRATION (MEQ./L.)			ARTERIOVENOUS DIFFERENCE (MEQ./L.)	
		VENOUS			
	ARTERIAL	RESTING LEG	EXERCISING LEG	RESTING LEG	EXERCISING LEG
1	0.70	–	0.72	–	−0.02
2	0.41	0.47	0.47	−0.06	−0.06
3	0.64	0.68	0.72	−0.04	−0.08
4	0.39	0.40	0.40	−0.01	−0.01
5*	1.02	0.95	0.90	0.07	0.12
6*	0.49	0.55	0.50	−0.06	−0.01

*Fasted for 36 hours.

To determine the effect of the metabolic state of the body the arteriovenous UFA difference was also studied after fasting. The behavior of UFA and lactic acid after 36 hours of fasting is shown in Fig. 4. As is apparent there was in this case already at rest an extraction of UFA in both legs. All of the time during work the UFA concentration of the blood leaving the exercising leg is lower than in the blood from the resting leg. Even at maximal work with lactic acid above 5 mEq. per liter there is an extraction of UFA which continues after

resting for 30 minutes. At that time 12.5 Gm. of glucose was given intravenously as indicated in the figure. Ten minutes later the arterial UFA concentration was reduced from 1.25 mEq. per liter to 0.51 mEq. per liter and the arteriovenous difference reversed.

DISCUSSION

The movement and metabolism of the unesterified fatty acids can schematically be considered to proceed as follows: fat depots → plasma → tissue cells → $CO_2 + H_2O$ where all steps except the last occur in both directions.[20] The plasma concentration of UFA is increased during fasting,[13] after epinephrine administration,[5, 12] in diabetic acidosis[13, 21] as well as in the "controlled" diabetic state,[21] in the hyperthyroid state,[22] and after administration of growth hormone.[23] A decrease has been found to occur after administration of glucose,[5] amino acids,[24] glucagon,[21] and insulin.[12] All these findings have been interpreted to mean that UFA is a major link in the transport of fat from the fat depots to the peripheral cells and that the status of the energy metabolism in some way controls the movement of UFA. It has been shown that epinephrine[25] as well as norepinephrine[26] accelerates the release of UFA from adipose tissues in vitro probably by accelerating lipolytic enzymes.[27, 28] Insulin on the other hand seems to decrease the release of UFA from the depots without affecting the removal rate from plasma.[29]

Our finding of a decreased systemic plasma concentration of UFA during exercise with an increase after a resting period of 30 minutes is of interest in view of our present knowledge of UFA as discussed above. The decrease could be due to either a reduced input or an accelerated outflow, or both. Studies on the blood sugar during the exercise has ruled out any hyperglycemic cause to the decreased UFA level. It is well worth considering in this connection that the urinary excretion of catechol amines[30, 31] as well as the plasma concentration of epinephrine and norepinephrine[32] is increased during exercise. This discrepancy between the increased catechol amine production and the decreased UFA level might be explained by an increased plasma removal of the unesterified fatty acids to meet the energy demand of the exercising muscles. The arteriovenous difference data lend further support to this hypothesis. Before discussing the possible significance of these data some points have to be considered.

The venous blood sampled drains not only muscular tissue but also adipose and cutaneous tissues. The venous level of UFA at rest generally was about 0.1 mEq. per liter higher than the arterial level, which is of the same magnitude as reported by Gordon and associates,[5] from analysis of plasma from the superior and inferior vena cava. However, when blood from the saphenous vein was collected a considerably higher negative arteriovenous difference was usually observed.[24] The negative arteriovenous UFA difference was supposed to be indicative of a mobilization of UFA from the fat depots. Furthermore, this difference could be abolished by administration of glucose and insulin. In our study the tip of the catheters have, in all probability, been situated distal to the saphenous vein. This could explain our lower venous UFA concentration

at rest in comparison to those of Gordon[24] as the blood we collected had drained fat depots to a smaller extent than the blood obtained from the saphenous vein.

One further point of importance is the relationship between the arterial and venous hematocrits. At rest the arterial and venous hematocrits were found to be equal. During exercise, however, the hematocrits differed from time to time. Sometimes the arterial and venous hematocrits were equal; sometimes the venous value was 1 to 5 per cent higher than the arterial hematocrit. Since no plasma protein analyses were performed, we have not considered it justified to make any volume corrections of our data although it seems reasonable to assume that the hematocrit changes, at least partly, were due to the chloride shift. If corrected for the increase of hematocrit, there will be an increase of the observed arteriovenous UFA difference.

The data of this study show that at moderate exercise unesterified fatty acids were extracted from the plasma perfusing the exercising leg. This taken together with the decreased systemic concentration seems to indicate an increased flow of UFA from the plasma into the muscular tissues. There is no proof that the extracted UFA were oxidized in the muscles. It was earlier believed that only the liver was capable of the first oxidative steps in the breakdown of the fatty acids. The formed ketone bodies on the other hand were used in the extrahepatic catabolism of fat. The view has now changed since it was shown in vitro that muscular and other tisssues could metabolize long chain fatty acids completely when added to slices either as triglycerides, fatty acid salts, or in the form of serum lipids.[33-35] This oxidation of fatty acids was also shown to occur in vivo.[36]

From this it follows that the observed extraction of UFA during exercise well might be due to the metabolism of the fatty acids to yield energy via the Krebs cycle. If we consider the extraction to be 0.1 mEq. per liter of plasma it can be calculated from the arteriovenous oxygen difference data[37] that more than 25 per cent of the oxygen utilized in the exercising leg would be required for the complete oxidation of the extracted UFA. Now it must be remembered that the observed extraction rates of UFA in all probability are minimum values as the venous UFA concentration probably has been increased by fatty acids delivered from the depots (cf. the resting values). Furthermore, the chloride shift will operate in the same direction. In conclusion, it can be said that if the extracted UFA are utilized in the muscular tissues and completely oxidized, a good deal of the energy during moderate exercise is derived from the combustion of fat, a view which is on a par with earlier findings in the beating heart[2, 4] and also consonant with metabolic studies on resting muscles.[3]

The reversal of the arteriovenous UFA difference in a leg starting to exercise from the resting state lends further support to the theory that UFA is one of the major transport forms for fat needed for the immediate energy metabolism.

The venous UFA concentration also decreased in the resting leg, leading to an abolishment of the initially negative arteriovenous difference. No unequivocal interpretation can be given for this change. It should be pointed out, however, that it was impossible to keep this leg completely resting. Of

further interest is the finding that the arteriovenous oxygen difference in the resting leg closely followed that of the exercising leg.[37] This finding suggested that the blood flow through the resting leg was decreased in order to allow a greater part of the circulating blood volume to pass through the exercising leg. The decreased venous UFA concentration in the resting leg could indicate a decreased flow of UFA from the adipose tissues. As discussed above there is no reason to believe that the output of UFA from the adipose tissues per se is decreased. It seems more likely that the decreased flow of UFA was to be ascribed to a reduced blood flow through the subcutaneous tissues. This possibility is also in good agreement with the observation that the venous oxygen concentration drops in the resting leg during exercise.

At maximal exercise the data seem to indicate that the extraction of UFA had diminished or ceased. Since the work was performed until the subject had performed his utmost, the blood flow through the leg certainly was insufficient to supply enough oxygen to maintain a completely aerobic metabolism. The metabolic pathways might at this point be directed toward the combustion of carbohydrates as an oxygen sparing mechanism. The increase of lactic acid at this point gives further support to this hypothesis.

In this study no significant difference in extraction of UFA could be demonstrated after fasting for 24 hours, but certainly the material is too small to permit valid conclusions in this direction. The immediate effect of glucose administration shown in Fig. 4 with a considerable decrease of arterial UFA concentration and a reversal of the arteriovenous UFA difference is on a par with earlier findings. When an excessive amount of glucose is made available the tissues need no longer extract unesterified fatty acids, and the mobilization from the depots is decreased.[5, 12, 24]

SUMMARY

The plasma concentration of unesterified fatty acids in the arterial blood and in the femoral veins was determined at rest and during exercise in 6 healthy subjects. During exercise the arterial plasma concentration decreased. The exercising leg extracted unesterified fatty acids from plasma. The findings have been discussed in relation to the energy metabolism of exercising muscular tissues.

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THE EFFECT OF A HIGH-SODIUM INTAKE ON THE DEVELOPMENT OF PERMANENT NEPHROSCLEROTIC HYPERTENSION

Effect of Nephrosclerotic Hypertension on the Granularity of the Juxtaglomerular Cells

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IN THE rat a partial constriction of one renal artery with a clip will result in a moderate hypertension in at least half of the operated animals. If the clipped kidney is excised within two months, the blood pressure promptly returns to normal levels. However, if the clip is allowed to remain on for 6 months, excision of the clipped kidney at this time will usually lower the blood pressure somewhat, but very often to levels which are still within the hypertensive range. Under these conditions, the rat will remain hypertensive indefinitely, even though his one remaining kidney has never been surgically manipulated in any way. It has been suggested, but by no means proved, that the nephrosclerotic changes in this one remaining kidney are responsible for the continuing hypertension.[1] We wanted to know whether a high-sodium intake would increase the incidence of this permanent "nephrosclerotic" hypertension, which continues after excision of a clipped kidney.

We were also interested in comparing the granulation of the renal juxtaglomerular cells in rats with this permanent "nephrosclerotic" hypertension with that in other rats whose blood pressure returned to normal levels after excision of the clipped kidney. In various other forms of experimental hypertension, a kidney that is exposed to high arterial pressure usually has a decreased granularity of the juxtaglomerular cells. In desoxycorticosterone[2, 3] and postdesoxycorticosterone[3] hypertension, the juxtaglomerular cells are significantly degranulated. This is also true for adrenal regeneration hypertension[3] and for the "untouched" kidney in unilateral renal hypertension.[2, 3] The work of Floyer[4] would indicate that the lone remaining "untouched" kidney plays a part in permanent "nephrosclerotic" hypertension. Hence, it seemed of interest to assess the granularity of its juxtaglomerular cells.

METHODS

A silver clip was used to partially constrict one renal artery of a large number of male Wistar rats. Starting immediately after the operation, one group of these rats was allowed to drink only a 1 per cent sodium chloride solution. Another group of the operated rats was allowed to drink only tap water. Both groups were eating Purina laboratory

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chow. Roughly two-thirds of the rats in either group became hypertensive, with a blood pressure level over 140 mm. Hg. Six months after the initial clipping procedure, the clipped kidney in the hypertensive rats with blood pressures over 145 in both groups was excised, and the rats were observed for 6 more weeks. During this final 6-week period, both groups drank tap water. Several blood pressure determinations were made during the 2-week period before excision of the clipped kidney, and again during the final week of the 6-week period following excision of the clipped kidney. The average of these blood pressure determinations was used to determine the true blood pressure before nephrectomy and the true blood pressure near the end of the 6 weeks following the nephrectomy. At the end of the 6 weeks following the nephrectomy, the rats were killed and the one remaining kidney was fixed in Helly's fluid and stained with Bowie's stain in the manner described by the Hartrofts.[2] The granulation of juxtaglomerular cells was then estimated using their method. The details of this procedure, as used in our laboratory, are outlined in a previous publication.[3] The mean juxtaglomerular index of a normal adult Wistar rat is 35. The blood pressures were determined with the Friedman microphonic method.[5] The mean blood pressure of a normal male Wistar rat, 6 months of age is 111 mm. Hg (S.D. $= \pm$ 9 mm. Hg) with this method. A rat was considered to be definitely hypertensive if it had a blood pressure of 140 or over, a level more than 3 standard deviations above the mean for normal rats. If it had a pressure below 126 mm. Hg, it was considered fairly normotensive. The blood pressures between 126 and 139 were intermediate and probably represented very mild hypertension. All the rats in this study were about 9 months old at the time they were killed.

Still another group of rats with a clip on one renal artery, whose blood pressure had always remained normotensive, underwent an excision of the clipped kidney 6 months after the clipping procedure. The rats in this group were observed for 6 weeks after the removal of the clipped kidney and were then killed. Their one remaining kidney was also examined for juxtaglomerular granulation to compare with that of the two preceding groups.

In describing the results, Floyer's useful terminology is employed. The "clipped" kidney is one with the renal artery partially constricted; the "untouched" kidney is the unmanipulated kidney opposite to the "clipped" one.[4]

RESULTS

Table I indicates the fate of the rats drinking either salt solution or tap water, after the clipping of one renal artery. At both 4½ and 6 months after the clipping, the per cent of rats in both groups which became hypertensive is quite similar. A slightly higher percentage of rats drinking tap water failed to reach hypertensive levels. The rats drinking saline solution had a slightly greater mortality rate. These are all small differences, however. For a statistical analysis of the percentage of rats becoming hypertensive, we analyzed the data obtained 4½ months following clipping, since the mortality was not very great at this time. The difference between the group drinking salt water and the group drinking tap water was not significant ($p = 0.4$). The mortality rates at both 4½ and 6 months were also analyzed statistically, and the groups have no significant differences ($p = 0.3$). The similar results in both groups indicate that a higher salt intake of this degree does not accelerate the development of this type of hypertension. Needless to say, this was contrary to our expectations.

Every rat with a blood pressure over 145 mm. Hg in the two groups described above underwent an excision of the "clipped" kidney. From then on,

TABLE I. BLOOD PRESSURE AND MORTALITY OF RATS WITH A CLIP ON ONE RENAL ARTERY DRINKING EITHER SALINE OR TAP WATER

GROUP	NO. OF RATS AT BEGINNING OF EXPERIMENT	PER CENT OF RATS WITH B.P. BELOW 140 MM. HG.	PER CENT OF RATS WITH B.P. 140-159 MM. HG.	PER CENT OF RATS WITH B.P. ABOVE 160 MM. HG.	PER CENT OF RATS THAT ARE DEAD
A. Observations 4½ mo. after clipping one renal artery:					
a. Rats drinking 1 per cent saline	63	27	8	49	16
b. Rats drinking tap water	52	35	8	44	13
B. Observations 6 mo. after clipping one renal artery:					
a. Rats drinking 1 per cent saline	63	19	17	38	25
b. Rats drinking tap water	52	29	8	46	17

TABLE II. CHANGES IN THE BLOOD PRESSURE OF INITIALLY HYPERTENSIVE RATS FOLLOWING REMOVAL OF AN "ISCHEMIC" KIDNEY

GROUP	NUMBER OF RATS IN GROUP	PER CENT OF GROUP WITH B.P. 140 MM. HG OR ABOVE 6 WK. AFTER NEPHRECTOMY	PER CENT OF GROUP WITH B.P. BETWEEN 126 AND 139 MM. HG 6 WK. AFTER NEPHRECTOMY	PER CENT OF GROUP WITH B.P. 125 MM. HG OR LOWER 6 WK. AFTER NEPHRECTOMY
Hypertensive rats previously drinking saline	28 (Average B.P. 178)	32 (Average B.P. 164)	32 (Average B.P. 133)	36 (Average B.P. 118)
Hypertensive rats previously drinking tap water	23 (Average B.P. 178)	43 (Average B.P. 158)	39 (Average B.P. 134)	17 (Average B.P. 111)

TABLE III. JUXTAGLOMERULAR GRANULATION IN THE REMAINING KIDNEY OF HYPERTENSIVE AND NORMOTENSIVE GROUPS OF RATS 6 WEEKS AFTER EXCISION OF AN "ISCHEMIC" KIDNEY

GROUP	NUMBER OF RATS	MEAN BLOOD PRESSURE 6 WK. AFTER EXCISION OF "ISCHEMIC" KIDNEY (MM. HG)	DATA CONCERNING JG* INDICES		
			MEAN	RANGE OF VALUES	STANDARD ERROR OF THE MEAN
A. Permanently hypertensive rats (above 140 mm. Hg)	13	159	8.5	1-17	±1.3
B. Previously hypertensive rats that became normotensive following removal of a "clipped" kidney	14	116	18.6	5-43	±2.6
C. Rats that remained normotensive both after the "clipping" of one renal artery and after the excision of this "clipped" kidney 6 mo. later	15	111	18.3	10-29	±1.4

*JG = Juxtaglomerular.

and for the next 6 weeks, all the rats in both groups drank only tap water. In Table II, the response of the blood pressure following excision of the "clipped" kidney is shown. Before the nephrectomy, both the group drinking saline and the group drinking tap water had identical average blood pressures of 178 mm. Hg. Six weeks after the nephrectomy, the rats which had previously been drinking saline had slightly less permanent hypertension and considerably more normal blood pressures than the group which had always been on tap water. However, these differences were not statistically significant. Here again, the drinking of a 1 per cent saline solution for 6 months did not increase the incidence of this permanent hypertension. If this permanent type of hypertension is related to the nephrosclerotic lesions which always occur in the "untouched" kidney, we can conclude that the 1 per cent saline did not accelerate the development of these lesions. However, there may be factors in the causation of this permanent hypertension which are unrelated to the nephrosclerotic lesions. In either group, the rats with the most severe hypertension were more apt to retain a permanent hypertension after removal of the clipped kidney.

The granulation of the juxtaglomerular cells was determined in the remaining "untouched" kidney of both hypertensive and normotensive rats 6 weeks after the excision of the clipped kidney (as described above). Previous studies have indicated that the index of juxtaglomerular granulation is between 0 and 4 in this "untouched" kidney when the "clipped" kidney is still present.[3] This is also true for rats that have gone 6 months since the original clipping procedure. For purposes of comparison (see Table III), the rats were divided into 3 groups. Group A consisted of rats whose blood pressure had been above 145 before and remained above 140 mm. Hg 6 weeks after excision of the clipped kidney. Group B contains rats whose blood pressure changed from above 145 before to below 125 mm. Hg after the excision of the clipped kidney. Group C consists of rats that remained normotensive 6 months after the original clipping and continued to be normotensive 6 weeks after the removal of the clipped kidney. The permanently hypertensive rats of Group A had a mean juxtaglomerular index of 8.5 with a range of 1 to 17. This is a highly significant degree of degranulation when compared with either of the normotensive groups B and C ($p = 0.0005$).

DISCUSSION

The data here bring out very clearly a fundamental difference between the hypertension produced by a partial constriction of one renal artery, and that produced by large doses of desoxycorticosterone, or by the presence of regenerating adrenal tissue. The drinking of 1 per cent saline solution produced no appreciable increase in the incidence and severity of this type of renal hypertension, whereas it greatly enhances desoxycorticosterone hypertension[6] and the hypertension following adrenal enucleation or adrenal pedicle ligation.[7, 8]

The exact cause of permanent "nephrosclerotic" hypertension is not known with certainty. The "untouched" kidney of rats with hypertension following partial constriction of one renal artery nearly always has some lesions of the renal arteries and arterioles.[1] These lesions undoubtedly contribute to the permanent hypertension which may remain after excision of the "clipped" kidney. However, we have seen this permanent hypertension in rats with minimal lesions in the remaining "untouched" kidney. Such minimal lesions are no more severe than those seen in many rats whose blood pressure returned to normal after excision of the "clipped" kidney. This finding has also been reported by Friedman and associates.[9] Rats of this type definitely bring up the possibility that there are factors other than the nephrosclerotic lesions which are responsible for the permanent hypertension. What ever its various causes, it would appear from our data that the drinking of 1 per cent saline for 6 months while the hypertension is developing does not accelerate the process or processes involved.

It is interesting to compare the juxtaglomerular granulation in the rats with permanent "nephrosclerotic" hypertension with the granulation in hypertensive rats whose "clipped" kidney is excised 7 weeks after the original clipping operation. In a previous study, it was found that the "untouched" kidney in hypertensive rats 7 weeks after clipping was almost completely degranulated with a mean juxtaglomerular index of 1 and a range of 0 to 4.[3] If the "clipped" kidney is removed at this time, the hypertension rapidly and completely disappears in every rat. Six weeks after the removal of the clipped kidney, the blood pressure in every one of 11 rats was normal, and the juxtaglomerular index of the remaining "untouched" kidney had increased to a mean value of 28 with a range of 16 to 39.[3] When the blood pressures were restored to normal in that study, the granulation of the juxtaglomerular cells returned to normal levels. If the excision of the "clipped" kidney is deferred for 6 months as in the present study, the results are somewhat different. When the "clipped" kidney is removed at this time, some regranulation of the juxtaglomerular cells does occur. If the rat remains permanently hypertensive, the regranulation is relatively slight, so that a mean juxtaglomerular index of 8.5 is attained. If the blood pressure of the rat returns to normal levels after excision of the "clipped" kidney, the regranulation is fairly extensive, ending up with a mean index of 19. This was also true for the rats which had always been normotensive. The question naturally arises as to why these rats who had regained normal or almost normal blood pressures did not increase their juxtaglomerular granulation to normal levels of around 35. It is possible that the prolonged period of degranulation of the juxtaglomerular cells in these rats permanently altered them in such a way as to prevent the subsequent attainment of normal granularity. This explanation would also apply to the rats which had always been fairly normotensive, since, they too, have extensive degranulation of the "untouched" kidney.[3]

The granulation in the hypertensive rats (Group A) was significantly lower than in either group of comparable normotensive rats (Groups B and C, Table III). Here we have another demonstration that high perfusion pressures in the kidney are associated with relative degranulation of the juxtaglomerular cells. The significance of this has been discussed previously.[3]

It is not certain how these findings may be related to human hypertension. In permanent "nephrosclerotic" hypertension, the lone remaining kidney has never been surgically manipulated in any way. One cannot be absolutely certain that this is actually a "renal" hypertension, although the studies of Floyer[4] indicate that if the original clip itself is removed, the continued presence of the "untouched" kidney tends to perpetuate the hypertension, while the removal of this "untouched" kidney promotes a return to normal blood pressures. Of all the various forms of experimental hypertension, this permanent "nephrosclerotic" hypertension and postdesoxycorticosterone hypertension are the most comparable to human hypertension. They are, therefore, particularly valuable for experimental studies.

SUMMARY

Partial constriction of one renal artery in rats will produce hypertension in 50 per cent or more of them. The incidence of hypertension is not significantly increased if the rats drink a 1 per cent sodium chloride solution instead of tap water. If the ischemic "clipped" kidney is excised 6 months after the initial clipping procedure, hypertension will persist indefinitely in some rats and disappear in others. Hypertension of at least a moderate degree persisted indefinitely in 32 per cent of rats that drank only 1 per cent saline for the 6 months preceding excision of the "clipped" kidney, whereas it persisted indefinitely in 43 per cent of rats drinking only tap water for this same period. Thus, the drinking of the 1 per cent salt solution did not increase the incidence of this permanent "nephrosclerotic" hypertension. The juxtaglomerular granulation index averaged 8.5 in the lone remaining kidney of a group of these permanently hypertensive rats drinking only tap water, whereas it averaged 18.6 in the lone kidney of previously hypertensive rats whose hypertension had disappeared after excision of the ischemic "clipped" kidney ($p = 0.0005$). The juxtaglomerular granulation index averaged 18.3 in a group of rats that remained normotensive after the clipping of one renal artery and after the excision of the clipped kidney 6 months later. Thus, the rats with permanent "nephrosclerotic" hypertension have a significantly decreased granularity of the juxtaglomerular cells when compared to either of the two normotensive control groups.

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THE ROLE OF PERIPHERAL VENOUS CONGESTION IN THE MAINTENANCE OF SUSTAINED PLASMA QUINIDINE LEVEL

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CLINICAL experience has shown that administration of quinidine is more hazardous in patients with congestive heart failure than in those without failure.[1-5] This observation has gained objective support by the demonstration of sustained plasma level of quinidine in the presence of congestive heart failure.[6-9] The failing heart muscle and the congested liver and kidney have been suspected to play a part in the causation of this phenomenon.[7, 10] However, we were unable to find any clinical or experimental proof which could demonstrate the factor responsible for the slow disappearance of quinidine from the plasma of patients with congestive heart failure.

In this study we have attempted to make experimental observations on the role of passive congestion of the liver, kidney, or the more peripheral venous system of the dog in the maintenance of the plasma quinidine level.

MATERIALS AND METHODS

Thirty-two experiments were performed on 18 mongrel dogs, weighing between 10 and 18 kilograms. In 4 dogs the thoracic inferior vena cava, in 4 dogs the abdominal vena cava above the renal veins, and in 5 dogs the abdominal vena cava below the renal veins were constricted by ligatures to about one fourth of their original diameters; this was done 7 to 21 days prior to the experiment. Five normal and apparently healthy dogs served as controls.

Anesthesia was induced by intravenous administration of 15 mg. sodium thiopentone (Pentothal*) per kilogram of weight, and light anesthesia was maintained for 3 hours by administration of the same drug, as needed. The total amount of the anesthetic agent was approximately the same in all dogs, ranging between 25 to 35 mg. per kilogram. Quinidine sulfate (10 mg. per kilogram) was administered intravenously during a period of 5 minutes. Blood samples were withdrawn 15, 60, 120, and 180 minutes after completing the injection, and an additional blood sample was taken 24 hours later from the unanesthetized animal. The plasma level of quinidine was determined in each sample by the Brodie-Udenfriend photofluorometric method,[11] using a Coleman apparatus. The amount of quinidine in the plasma was expressed in absolute values (milligrams per liter), as well as in percentage, taking the 15-minute sample value as a 100 per cent. In some instances liver and kidney biopsies were taken for histologic sections and the presence of passive congestion was proved.

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RESULTS

Group I. Controls.—Eight experiments were performed on 5 apparently healthy dogs. The average values of the various samples are summarized in Table I.

It may be seen that 60 minutes after the injection of quinidine an average of 71.5 per cent was present in the plasma when compared with the first sample; 120 and 180 minutes following the injection this value averaged 45.5 and 40.5 per cent, respectively. Twenty-four hours after the injection the average quinidine level was 8.0 per cent (Fig. 1).

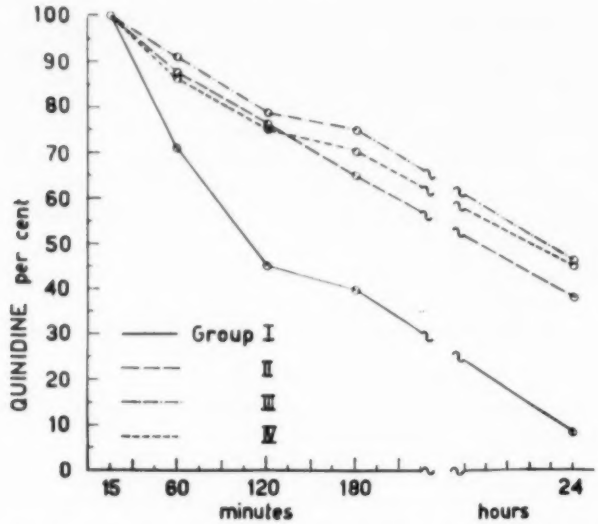

Fig. 1.—Plasma quinidine level 15 minutes, and 1, 2, 3, and 24 hours after intravenous administration of quinidine sulfate, expressed as per cent of the 15-minute sample value. Group I = control. Group II = constricted inferior vena cava above the hepatic veins. Group III = constricted inferior vena cava above the renal veins. Group IV = constricted vena cava below the renal veins.

Group II. Constricted Inferior Vena Cava Above the Hepatic Veins.—Eight experiments were performed on 4 dogs in which the thoracic vena cava was constricted above the entrance of the hepatic veins. This procedure caused marked congestion in the liver and ascites. The average levels of quinidine in the various samples are presented in Table I.

It can be seen that the disappearance of quinidine from the plasma was much slower in this group than in the controls (Fig. 1). One hundred and twenty minutes and 180 minutes following the injection, 77 and 66 per cent, respectively, and 24 hours later 39 per cent of the initial quinidine level was still detectable in the plasma. Statistical analysis of the results showed significant differences between these values and those obtained in the control group.

Group III. Constricted Inferior Vena Cava Above the Renal Veins.—Eight experiments were performed on 4 dogs, in which the inferior vena cava was constricted about 1 cm. above the renal veins, producing passive congestion of the kidneys and the more peripheral venous system. Table I summarizes the average values of quinidine obtained in the various blood samples.

It is noteworthy that the average disappearance rate of quinidine is practically identical in this group with that found in the group of dogs with congested liver (Fig. 1). Here again, significant differences exist between the results obtained in this group and those found in the control group, except for the 60-minute sample.

TABLE I. AVERAGE VALUES OF QUINIDINE LEVELS IN PLASMA*

GROUP	15 MINUTES	1 HOUR	2 HOURS	3 HOURS	24 HOURS
Group I (Control)	3.0 ± 0.28 (100)	2.15 ± 0.28 (71.5)	1.36 ± 0.51 (45.5)	1.22 ± 0.14 (40.5)	0.24 ± 0.31 (8)
Group II	3.60 ± 0.66 (100)	3.16 ± 0.78 (88) $p < 0.05$	2.76 ± 0.53 (77) $p = 0.001$	2.38 ± 0.52 (66) $p < 0.01$	1.40 ± 0.65 (39) $p = 0.001$
Group III	3.20 ± 1.08 (100)	2.93 ± 1.22 (91.5) $p > 0.1$	2.54 ± 0.99 (79) $p = 0.01$	2.41 ± 1.02 (75.5) $p = 0.05$	1.50 ± 0.72 (47) $p = 0.001$
Group IV	3.20 ± 0.62 (100)	2.79 ± 0.83 (87) $p > 0.1$	2.44 ± 0.73 (76.5) $p < 0.01$	2.27 ± 0.78 (71) $p < 0.02$	1.47 ± 0.94 (46) $p < 0.01$

*Following intravenous injection (10 mg. per kilogram) in milligrams per liter and as per cent of the 15-minute sample (in parentheses). The p values relate the respective values to the corresponding values in the control group.

Group IV. Constricted Inferior Vena Cava Below the Renal Veins.—Eight experiments were performed on 5 dogs, in which the inferior vena cava was constricted about 1 cm. below the entrance of the renal veins, thus producing congestion in the distal venous circulation. Table I shows the rate of disappearance of quinidine from the plasma.

The average values of plasma quinidine concentrations in this group closely resembled those found in the 2 latter groups, and differed significantly from the control group.

DISCUSSION

The slow disappearance of quinidine from the plasma of patients suffering from congestive heart failure has been previously demonstrated by a few investigators, although no satisfactory explanation has been provided.[6-10] In our series of dogs in which liver congestion was produced by constricting the thoracic inferior vena cava, we were able to demonstrate significantly delayed quinidine elimination when compared with the control group. It is evident from this experiment, that for the maintenance of sustained quinidine level the presence of a failing heart is not obligatory.

Since the liver is known to be the detoxifying organ, and in congestive heart failure its function may be disturbed by the engorgement, one could expect to find laboratory evidence proving that the congested liver is responsible

for the slow disappearance of quinidine from the plasma. Although this was not confirmed by Swisher and his associates,[7] since in their patients with liver failure but without congestive heart failure the plasma quinidine level was not unduly sustained, our results in a group of dogs with liver congestion seemed to provide proof for this assumption. However, this procedure caused congestion not only in the liver but in the whole venous system below the level of constriction; thus, we felt that the role of the liver as a single cause in the production of the phenomenon had not been proved. Therefore, we carried out experiments in a second series of dogs, in which engorgement of the kidneys and the venous system was similarly produced as in the previous group, but without congestion of the liver. It was surprising to find that in this group of dogs the rate of elimination of quinidine was similar to that found in dogs with liver congestion. This observation raised the possibility that the kidneys play a major role in the regulation of plasma quinidine levels. A number of investigators, however, have demonstrated that renal excretion is of minor importance in the disappearance rate of quinidine.[12-15] Furthermore, Lindseth Ditlefsen[10] has found similar amounts of quinidine in the urine of patients with and without congestive heart failure.

We performed experiments, therefore, on dogs in which the constriction of the vena cava was produced below the renal veins. In this group there was peripheral venous engorgement without congestion of the liver or kidneys. The rate of quinidine elimination in this group resembled closely that of the 2 latter groups; thus, in all 3 the plasma quinidine level was much more sustained than in the control group.

It would seem that the slow disappearance of quinidine from the plasma is due neither to the congestion of the liver nor to the congestion of the kidney, but may be explained by systemic venous engorgement alone. It may be assumed that the sustained quinidine level in patients with congestive heart failure is produced by a similar manner. The exact mechanism by which the peripheral venous congestion leads to slow disappearance of quinidine from the plasma is still to be clarified. It is possible, that impaired venous circulation causes a slow and prolonged release of the drug from the tissues, thus maintaining a sustained blood level of quinidine.

SUMMARY

In order to identify the organ responsible for the sustained plasma quinidine level in patients with congestive heart failure, experimental congestion of the liver, kidneys, and the more peripheral venous system was produced in dogs.

The elimination of quinidine from the plasma was studied by the determination of quinidine level 15 minutes, and 1, 2, 3, and 24 hours after the intravenous administration of quinidine sulfate. In the control group 3 hours after the injection 40.5 per cent and 24 hours later 8 per cent of the quinidine was detectable in the plasma when compared with the 15-minute sample. In groups of dogs in which the thoracic inferior vena cava, abdominal vena cava

above the renal veins, and abdominal vena cava below the renal veins were constricted 7 to 21 days prior to the experiment, the disappearance of quinidine was markedly delayed and, 24 hours after the injection, 39, 47, and 46 per cent of the initial level, respectively, was still present in the plasma.

It is assumed, therefore, that the main or perhaps only factor for sustained quinidine level in the plasma is the peripheral venous congestion and not the congested liver or kidneys. It is suggested that a similar mechanism may be responsible for sustained quinidine level in patients with congestive heart failure.

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LEUKOCYTE AGGLUTININS

I. Method of Detection and Clinical Observations

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INTEREST in the subject of leukocyte agglutinins has been heightened in recent years by the awareness of the probable role of these antibodies in transfusion reactions,[1-3] in leukopenic states,[4, 5] and by the suspicion that they may be related to humoral mechanisms which participate in some types of anemia and thrombocytopenia.[6]

Difficulties which we have encountered in obtaining reproducible results with various published techniques and the desirability of having available a simple test tube serologic method led us to develop another test for leukocyte-agglutinating antibodies. It is the purpose of this communication to describe this test and some of the observations based on it.

Many workers agree that the natural tendency of leukocytes in suspension to undergo spontaneous conglutination with resultant false-positive "agglutination" is a common and very imposing pitfall in this work.[7] Furthermore, with many of the techniques which have been described as false positive, leukoagglutinin tests may result from the agglutination of erythrocytes which contaminate the leukocyte suspensions employed as antigens. Because of uncertainties regarding the proper criteria for positive and negative tests, antisera to human leukocytes were prepared in rabbits and these served as the primary source of known positive controls.

These antisera were prepared as follows: 1.0 ml. of thrice-washed densely packed leukocyte suspension from a group O donor is added dropwise to 0.5 ml. of falba and 0.5 ml. of mineral oil at 65° C. Mixing is effected by pulling the material in and out of a small bore syringe. The leukocyte suspension in this quantity is most economically obtained by sedimentation of fresh "incomplete" blood bank donations.

After the initial bleeding of a 5-pound New Zealand white rabbit, 1.0 ml. of the leukocyte adjuvant mixture is injected intramuscularly into each thigh. Ten milliliters of venous blood or blood from the heart is drawn at weekly intervals thereafter for 4 weeks. The serum is collected and stored at –10° C. until tested.

Immediately before testing the serum is inactivated in a water bath at 65° C. for 15 minutes and adsorbed twice with equal volumes of group O

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erythrocytes in a water bath at 37° C. for 30 minutes. In all other particulars the technique of leukocyte agglutination is the same as that described here for human serum.

MATERIALS AND METHODS

Preparation of Antigens.—Fresh leukocyte suspensions are employed each day. Approximately 15 ml. of venous blood of a healthy group O donor is drawn into a syringe containing 0.5 ml. of 5 per cent disodium Versenate and 1.0 ml. 6 per cent dextran in physiologic saline. Siliconized glassware is used throughout. The blood is placed in three 200 by 15 mm. test tubes and allowed to settle for 1 to 2 hours at room temperature, after which the plasma suspension of platelets and leukocytes is removed. This suspension is distributed into two or three 120 by 15 mm. test tubes and centrifuged at 250 r.p.m. for 15 minutes. The supernate consisting of plasma and platelets is removed and the original volume is re-established by the addition of physiologic saline. By repeating this procedure the resulting leukocyte sediment is relatively free of platelets. After the second washing with physiologic saline, the leukocytes are finally resuspended in an amount of isotonic saline containing 2.5 per cent bovine albumin sufficient to effect a density of approximately 10,000 W.B.C. per cubic millimeter. Purity of the leukocyte suspension depends on the length of time the blood is permitted to settle. After 2 to 2½ hours of sedimentation, an almost pure leukocyte suspension with scarcely any erythrocyte contamination may be obtained; however, the leukocyte yield is reduced considerably since there is also an appreciable sedimentation of leukocytes. After 1½ hours of sedimentation the leukocyte-erythrocyte ratio is usually 1:1 or 1.5:1. However, even heavy erythrocyte contamination has not interfered significantly with this test.

Serum.—Test serum is obtained from clotted venous blood by the usual methods and stored in the frozen state. Prior to testing, an aliquot of the serum sample to be tested is inactivated by heating at 56° C. in a water bath for 30 minutes. Sera which are to be tested repeatedly are stored in small aliquots, since repeated freezing and thawing results in loss of intensity of the agglutination reaction.

Testing Procedure.—The agglutination test is performed in 10 by 75 mm. culture tubes. Constituents of the system may be rigidly quantitated with volumetric pipettes but we have found it convenient and satisfactory to use serologic dropper pipettes. Five dilutions (1:8 through 1:128) of the test serum in 4 drop quantities are prepared in isotonic saline; to these are now added two additional drops of saline and two drops of leukocyte suspension in the previously noted concentration of 10,000 W.B.C. per cubic millimeter. Known positive controls* and a negative control, composed of a pool of 6 normal sera, are included with each battery of tests. The tubes are shaken and placed in a Dubenoff-type shaking water bath at 37° C. for 30 minutes. After removal from the water bath and an additional incubation at room temperature for one hour, the tests are read. A second reading is necessary at the end of 2 hours since often positive reactions appear late in the incubation. The tubes must be read with appropriate transillumination and magnification of the suspensions. This is best obtained by holding the test tube over a small concave mirror (microscope substage mirror) and directly in a strong light beam such as that furnished by a standard high-intensity microscope lamp. Fig. 1 is a photograph of positive and negative tests as seen in the magnifying concave mirror.

In the procedure just described, fresh leukocytes from a group O donor are employed. Initially, O-Rh negative donors were used in order to satisfy theoretical objections to the possibility of the leukocytes being type-specifically antigenic.[9-12] In this test system, we have found no indication that washed leukocytes are identified with the ABO system. We were not able to demonstrate positive agglutination with normal group

*These positive controls were initially identified as "positive" by characteristic agglutination similar to that observed in the rabbit positive sera.

O serum and leukocytes from A, B, or AB donors. Conversely, 3 different group A sera, containing leukoagglutinins, did not produce differences in titers with leukocytes from A, B, AB, or O donors. If one accepts the group specificity of leukocytes, indicated by several recent studies,[9-12] one must assume that such a leukocyte-antibody reaction is either too weak to be apparent in the presence of bovine albumin or in the serum dilution employed here. In any instance, if the leukocytes are properly washed and are not too markedly contaminated with erythrocytes, a healthy donor of any blood group should prove satisfactory.

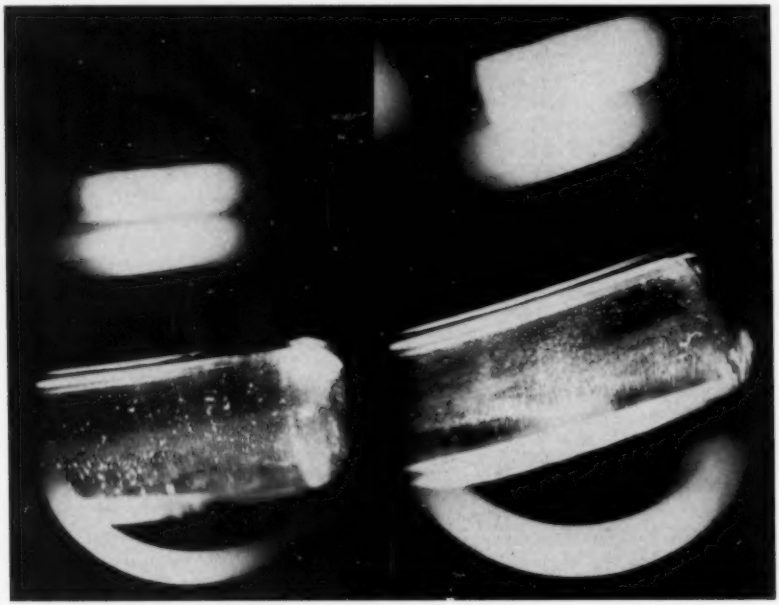


Fig. 1.—Positive (left) and negative (right) tests as seen reflected by the concave mirror.

A number of factors tend to produce false positive tests and will detract from the reproducibility of this test if certain precautions are not observed: (1) Fresh leukocytes are definitely preferable to stored cells. The latter tend to conglutinate and require more critical observation for the interpretation of the test. Similarly, storage of the cells in gelatin does not provide suitable suspensions for this technique. (2) Hyperglycemia, azotemia or any other severe debility of the donor seem to produce an alteration of granulocytes, which in turn tends to cause decreased ameboid activity and increases the tendency toward nonspecific agglutination. Hyperglobulinemia also tends to cause pseudo-agglutination. Therefore, healthy donors should routinely be used. (3) Saline suspensions of granulocytes tend to undergo nonspecific clumping on standing. The stabilizing effect of bovine albumin is necessary to maintain a suitable suspension of the fresh cells. In serial tests with varying concentrations of albumin, we have observed that 2.5 per cent albumin in isotonic saline furnishes the optimal physical factors for cell suspensions approximating 10,000 to 20,000 W.B.C. per cubic millimeter of density. (4) In order to avoid false positive reactions, serum dilutions of less than 1:8 are not included in the titrations. In doing this we may occasionally miss some weakly positive tests. (5) After more than 3 hours from the beginning of incubation, nonspecific agglutination develops, thus necessitating rigid adherence to the testing method just described.

RESULTS

The sera of 647 patients have been tested. Of these, 23 have been positive for leukocyte agglutinins in 2 or 3 tests, but have become negative after prolonged storage. Thirteen others have shown leukocyte agglutinins which are demonstrable on repeated testing and after prolonged storage. These are listed in Table I. It will be noted that 10 of the 13 cases exhibited blood dyscrasias of which 4 were monocytic or monoblastic leukemia, 3 were chronic lymphocytic leukemia, 2 were idiopathic pancytopenia (one with hypercellular, one with aplastic bone marrow), and one was a case of idiopathic thrombocytopenic purpura.

Ten of these 13 patients had received blood transfusions prior to the demonstration of serum leukocyte agglutinins. Four of these patients had received 3 units of blood or less, one had received several infusions of gamma globulin but no blood, and two had not received blood transfusions at any time.

Most of the patients with leukemia had received multiple blood transfusions prior to the detection of leukocyte agglutinins. Three of these had received 30 or more units, while one patient had received 17 and another had received 8 units of blood. However, one of these patients had received only two units of blood and one had never been transfused prior to testing; the latter patient had received nitrogen mustard therapy 6 years prior to the initial test for serum agglutinins.

One of the patients (Subject 13) who had received only one transfusion had entered the hospital because of an explosion burn involving 22 per cent of the body surface and received one unit of blood less than 12 hours before the first test serum specimen was drawn. The other patient (Subject 12), who received only one unit of blood prior to testing, received this 3 years previously; however, she had undergone two rather extensive abdominal operations for regional enteritis during this period and also suffered from chronic bronchitis and emphysema.

Both of the patients who developed leukocyte agglutinins in the apparent absence of transfusions exhibited hematologic disturbances. One of these (Subject 4) had a pancytopenia with an abundantly cellular marrow and was thought initially to represent primary hypersplenism. He died of a coronary occlusion 4 days postsplenectomy and microscopic examination of the spleen leaves some doubt as to the mechanism of the pancytopenia. The other patient (Subject 1) had chronic lymphocytic leukemia and had received treatment with nitrogen mustards 6 years previously as noted above. Subject 11 has been identified as having hypogammaglobulinemia and had received numerous infusions of gamma globulin as a prophylactic measure against recurrent furunculosis.

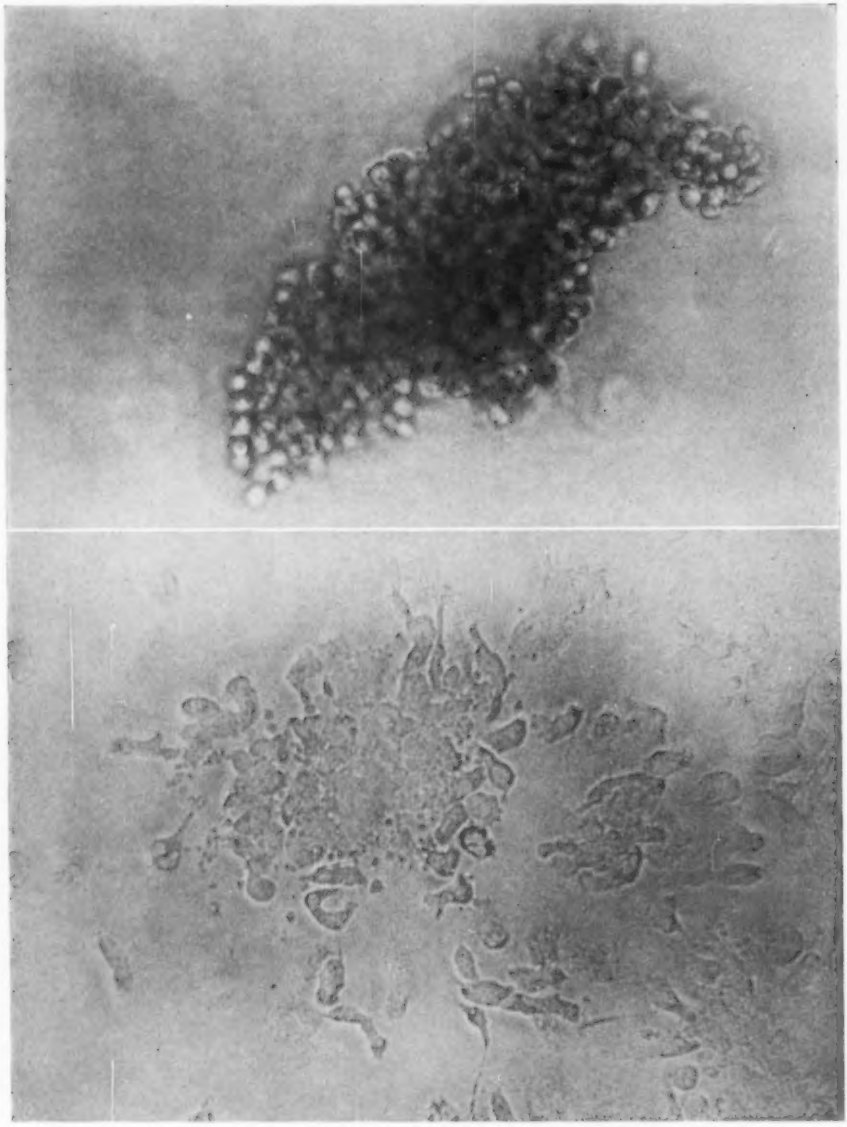
Seven of the 13 patients exhibited a pancytopenia at the time they were first seen in the clinic. All of these patients had leukemia or refractory bone marrow failure of uncertain etiology. The one patient with idiopathic thrombocytopenic purpura and leukocyte agglutinins exhibited leukocytosis as high

TABLE I

PATIENT	AGE AND SEX	DIAGNOSIS	PREVIOUS MEDICATIONS AND TRANSFUSIONS	LEUKOCYTE AGGLUTININS	ERYTHROCYTE AGGLUTININS	BLOOD CELL LEVELS
1. O. D. 644873	80 M	Chronic lymphocytic leukemia (''aleukemic'')	Nitrogen mustard therapy 6 years prior to testing	Pos. 1:16	Neg.	Pancytopenia leukopenia (2,000)
2. W. B. 628560	60 M	Chronic lymphocytic leukemia	8 units of blood prior to initial test, P^{32} therapy, x-irradiation, chlorambucil therapy	Pos. 1:32	Positive Coombs' direct and indirect, strong anti-C	Leukopenia (500-1,300), hemolytic anemia
3. J. M. (Ohio Pen.)	62 M	Chronic lymphocytic leukemia	50 units of blood prior to testing, P^{32}	Pos. 1:128	Neg.	Leukocytosis
4. F. K. 630952	60 M	Primary refractory pancytopenia with hyperplastic marrow	None	Pos. 1:64	Neg.	Pancytopenia
5. L. C. 542396	57 F	Pan marrow hypoplasia	50 transfusions	Pos. 1:128	Neg. Cold agglutinins, secondary hypersplenic, hemolytic anemia	Pancytopenia
6. L. W. 625339	26 F	Idiopathic thrombocytopenic purpura, healed pulmonary tuberculosis	3 units of blood	Became positive after splenectomy and stopping steroids	Neg.	Leukocytosis, thrombocytopenia, good response to splenectomy

7.	P. W. 590498	31	M	Monocytic leukemia, peptic ulcer hepatitis	33 units of blood Aminopterin 6-mercaptopurine corticosteroids	Pos. 1:64	Neg.	Leukopenia before treatment and since
8.	F. M. 645049	57	M	Monoblastic leukemia	2 units of blood	Pos. 1:16	Neg.	Pancytopenia
9.	A. W.	41	M	Monocytic leukemia	17 units of blood two weeks prior to testing 6-mercaptopurine	Pos. 1:16	Neg.	Pancytopenia
10.	F. F. 624036	46	M	Monocytic leukemia	30 units of blood	Pos. 1:32	Neg.	Pancytopenia
11.	D. F. 557485	30	F	Hypogammaglobulinemia, recurrent infections, coagulation defect	Plasma infusions	Pos. 1:64	Neg.	Slight leukocytosis
12.	G. N. 540528	36	F	Regional enteritis enterocolostomy, chronic bronchitis	1 unit of blood	Pos. 1:16	Neg.	Normal
13.	C. L. 622738	58	M	Explosion burn	1 unit whole blood 12 hours prior to testing	Pos. 1:16	Neg.	Relative and absolute granulocytosis

A.



B.

Fig. 2.—*A*, Case 5. Agglutination of large mass of leukocytes with disappearance of pseudopods. *B*, Leukocyte agglutination by serum which had been previously adsorbed with leukocytes. Note the free and active pseudopods despite agglutination of cells.

as 18,000 per cubic millimeter. The agglutinins were not demonstrated until after corticosteroid medication was stopped, one week postsplenectomy. However, serum leukoagglutinins were present 4½ months later.

Only 1 of the 13 patients exhibited erythrocyte agglutinins (other than blood group antibodies) demonstrated by the usual serologic techniques. Subject 2 had a strongly positive direct and indirect Coombs' test and showed a strong anti-C agglutinin. A number of other patients with leukemia appeared to have a shortened erythrocyte survival time in the absence of demonstrable complete or incomplete antibodies. One patient with pan-marrow hypoplasia (Subject 5) developed splenomegaly after approximately 30 transfusions. Following splenectomy, the chromium^{51}-tagged erythrocyte half-survival time rose from 8.5 to 22.5 days.

In Vitro Effect of Leukocyte Agglutinins.—The primary effect of leukocyte agglutinin activity is that of clumping of leukocytes in groups of 10 to several hundred cells per clump. Vital smears prepared from tube suspensions as described above reveal tight clumping of the cells with disappearance of the pseudopods (Fig. 2, *A*). The agonal changes which normally occur as result of cell contact with glass surfaces (hydropsical swelling, Brownian hypermotility, vacuolization) are probably accelerated by agglutination, but are of themselves significant only of cell death. Efforts to adsorb the leukoagglutinin activity of the serum from Subject 5 led to the detection of another type of agglutinating activity. Repeated incubation of a small aliquot (0.9 ml.) of serum with 3 successive equal volumes of washed leukocytes decreased the titer of the agglutination by only two tubes when the adsorbed serum was retested according to the routine technique. However, the adsorbed serum was still strongly positive in a dilution of 1:32. The character of the agglutination with this adsorbed serum was macroscopically different from that seen in a similar dilution of the untreated serum; the cell clumps appeared less discrete. Microscopic examination of these agglutinated cell clumps revealed that while there was still strong agglutination of the cells, the cytoplasmic pseudopods were free (Fig. 2, *B*). Indeed, the conglutination of the nuclei appeared to have stimulated increased pseudopod activity. These phenomena suggest that presence of separate agglutinins, distinctively specific for cytoplasm and for nuclei, thus offering the human counterpart of the agglutinins produced experimentally in rabbits by Miescher.[13]

Four of the patients in this series were observed to have repeated and severe transfusion reactions. Sedimentation of the buffy coat by slow centrifugation, followed by standing for 3 hours has made possible the siphon-removal of a sufficiently large proportion of leukocytes (and plasma) to obviate the reactions in all but Subject 5. This individual tolerates only washed and resuspended red cells without serious reactions.

DISCUSSION

The 23 cases with transiently positive sera were not discussed in detail because the transitory nature of the agglutinins precluded their further study.

However, the observations to date suggest that some antibodies in the leukocyte agglutinin category may disappear with storage.

Eight of the 13 persistently positive cases are other additions to the accumulating number of cases of leukocyte agglutinins induced by repeated blood transfusion. These are clearly isoantibodies. Since the majority of cases in this series, as in most others, have severe leukopenia it is difficult to test for leukocyte autoantibodies. However, incomplete antibodies which may be commonly associated with leukopenic and leukotoxic states have been demonstrated by van Loghem.[14] (This test, which we have repeated, requires an imposing quantity of the leukocyte antigen.)

It is noteworthy that other factors such as an extensive burn and multiple surgical procedures are also associated with representative cases in this series and suggests the possibility that extensive tissue damage may participate in the induction of this antibody response.

The commonest single group of diseases associated with strong leukocyte agglutinins in this small series was the leukemias. While only 91 leukemic patients have been tested thus far, 7 have shown strong leukocyte agglutinins, 4 of 14 monocytic leukemias, and 3 of 35 chronic lymphocytic leukemias are positive, but none of the 30 cases of chronic granulocytic leukemia tested have exhibited any evidence of agglutinins. Whether this discrepancy represents an acceleration or perversion of antibody response in monocytic and lymphocytic disease, or a suppression of antibody producing functions in granulocytic disease remains an open question at this time.

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LEUKOCYTE AGGLUTININS

II. Electrophoretic Separation of the Agglutinating Fraction

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IN AN effort to identify the source of the leukocyte-agglutinating factors responsible for the phenomena described in the preceding paper, sera were fractionated in a continuous flow paper electrophoresis cell.* The fractions were identified by standard paper strip electrophoresis and were then dialyzed, lyophilized, and tested for leukocyte agglutinins, employing a slight modification of our standard procedure (see below).

MATERIALS AND METHODS

To 5.0 ml. of serum is added 5.0 ml. barbital buffer† and the mixture is placed in the sample feeder of a continuous flow paper electrophoresis cell,‡ with a current of 40 Ma. across the curtain. The addition of Acationox detergent,§ 1.0 ml. per liter of buffer, greatly enhances the separation of the fractions. The fractionation is continued for approximately 18 hours. The curtain is removed, blotted, dried quickly, and stained with bromphenol blue for 4 hours. The number of the tabs on the paper curtain corresponds to the test tubes which collect the fractions. The fractions, 10 to 12 ml. each, are dialyzed separately in viscose tubing against double distilled, demineralized water for 24 hours at 10° C. The water should be changed at least once during this process.

Following dialysis, the fractions are transferred to 200 by 25 mm. test tubes for lyophilization. If one wishes to pool fractions in order to test broad segments of the separation, they should be pooled at this stage prior to lyophilization. The fractions are freeze-dried overnight on a VirTis apparatus. It is important to shell-freeze the tubes before the specimens are allowed to freeze dry, since any thawing before complete drying has occurred will spoil the fraction. When totally dry, each fraction is removed from the vacuum and reconstituted to 2.0 ml. with physiologic saline. (At this stage, fractions may be frozen and stored if it is necessary to interrupt the procedure.)

Identification of fractions is carried out by standard paper strip electrophoresis by introducing 0.01 ml. of the fraction (or test serum) onto each paper strip. Electrophoresis is conducted for 4 hours at 26 Ma., after which the strips are quickly blotted, oven dried, and stained with bromphenol blue.

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†Diethyl barbituric acid 0.74 grain, 4.1 grains sodium diethyl barbiturate, dissolved in distilled water to 1.0 liter of solution, ionic strength 0.02, pH 8.6.

‡Spinco Model CP, Beckman Instruments, Inc., Belmont, Calif.

§Marketed by Scientific Products Division, American Hospital Supply Corporation.

Agglutination studies on the separated reconstituted fractions are performed according to the method described in the previous paper except that the test fractions are buffered with an equal volume of inactivated normal serum just before each dilution is added dropwise to the tubes. The initial serum dilution, therefore, is 1:4 rather than 1:8.

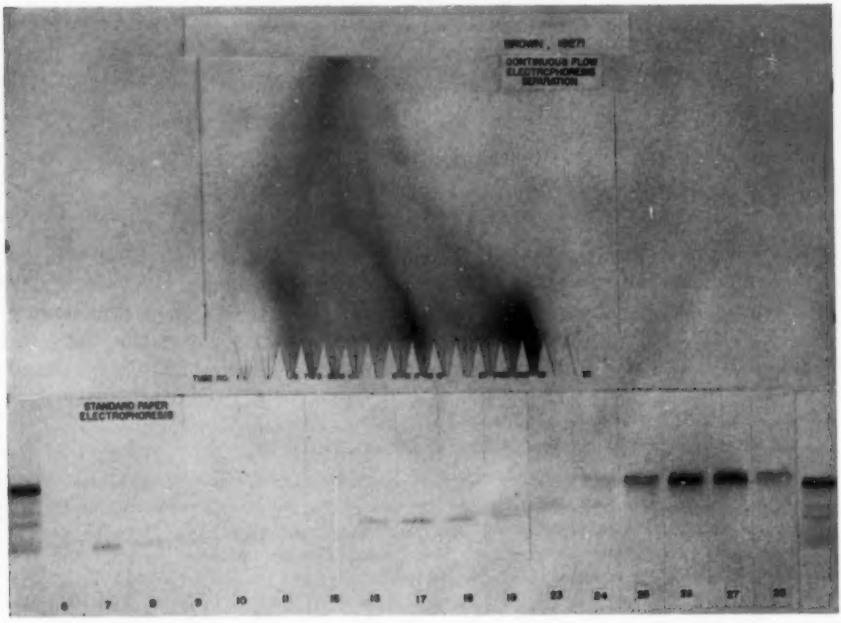

Fig. 1.—*Top*, Stained paper curtain of continuous flow electrophoresis cell. *Bottom*, Paper strips of the fractions obtained by standard electrophoresis. The numbers correspond to the paper tips on the curtain and the number of the fraction. All leukocyte agglutination activity was found in fractions 6 to 10 (gamma globulin).

RESULTS

Excellent separations of the serum fractions were obtained by this procedure. Fig. 1 shows the stained paper curtain of the continuous flow cell and beneath it the series of standard paper electrophoresis patterns which correspond to the fractions obtained. The fractions are numbered in series and correspond to the tips at the bottom of the paper curtain. The separation is usually sufficiently broad to distribute serum fractions into 28 tubes.

Seventeen sera have been fractionated and tested thus far for leukocyte agglutinins. Of these, 10 were positive and 7 were negative for leukocyte agglutinins when tested as whole serum. One of the 7 negative sera was a pool of normal sera from 6 donors, while another was from a patient with multiple myeloma, with an M-protein of gamma electrophoretic mobility. The latter serum was tested in an effort to determine whether physical factors inherent in a high nonspecific globulin might influence the agglutination reaction. In all of the 10 sera from 6 agglutinin-positive patients the activity is present

in the zone corresponding to the gamma globulins, but in no other fractions. Conversely, similar studies of the fractions of negative sera and those of pooled normal serum and of multiple myeloma failed to reveal any leukocyte agglutinating activity in any of the fractions obtained.

DISCUSSION

Tullis[1] has reported the occurrence of "leukotoxic" substances in the alpha globulin and beta globulin serum fractions, with a smaller amount of activity in the fast migrating material in front of albumin. He did not detect any activity in the gamma globulins. It seems clear that the leukocyte-agglutinating antibodies which we are describing here are distinct from the substances described by Tullis. We have made no attempt to isolate those apparently toxic factors from the sera of azotemic, febrile and debilitated patients, which tend to produce nonspecific pseudoagglutination. It is possible that these are in the category of the leukotoxic factors described by this author.

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HEMATOLOGIC AND COAGULATION STUDIES IN VARIOUS ANIMAL SPECIES

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IN STUDIES of the blood coagulation mechanism, it is often expedient to employ materials obtained from more than one animal species. In such mixed systems, however, problems of species specificity and species differences are important. Scattered throughout the literature are numerous reports of hematologic and coagulation tests in various animals.[1, 11] Some of the data presented in this paper are new; some have already appeared among the references just cited. This study, however, attempts to summarize facts concerning the coagulation mechanisms of 8 mammalian and 2 avian species, first using the methods currently in use in our laboratory for the study of human coagulation, and second employing, as far as possible, homologous species systems. Species specificities of tissue thromboplastins and of the thrombin-fibrinogen reaction were also investigated.

MATERIALS AND METHODS

Tissue Thromboplastins.—Brain powders were prepared from various animal brains by the acetone dehydration method of Quick.[2] The suspensions employed in the tests were prepared by gently mixing 300 mg. brain powder with 10 ml. imidazolesaline solution (pH 7.3, 1 part imidazole buffer[3] in 10 parts 0.85 per cent sodium chloride). Beef lung extract was prepared by the method of Ware and Seegers.[3]

Procedure.—The methods employed have been described in detail elsewhere[4, 5] or will be mentioned with the appropriate text. Owren's test for proconvertin[6] as used in these studies estimates the activities of two factors: factor VII or SPCA[6, 7] and Stuart factor.[8] In most species blood samples were obtained by cardiac puncture or venepuncture, employing a two siliconed syringe technique. Cow and sheep blood was obtained at the slaughterhouse by collecting free-flowing blood from a jugular gash into a siliconed beaker and transferring immediately to the appropriate tubes. Peripheral blood morphology was studied by both bright-light and phase contrast microscopy.

RESULTS

Table I illustrates the results of routine hematologic tests in the various species. The same 4 human subjects were used as controls on each test day and, for ease of comparison, levels of various factors in these subjects were designated as 100 per cent. Remarkable findings include the following: (1) Slow erythrocyte sedimentation rate in monkey, cow, rabbit, sheep, chicken, and duck,

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TABLE I. ROUTINE HEMATOLOGIC OBSERVATIONS
(Averages of All Determinations for Each Species)

SPECIES	MAN	MONKEY (RHESUS)	COW	SHEEP	DOG (MONGREL)	CAT (MONGREL)	RABBIT	RACCOON	OPOSSUM	CHICKEN (LEGHORN)	DUCK (PEKING)
Number of subjects	4	3	3	1	6	5	3	2	1	8	2
Number of determinations	11	3	3	1	10	8	3	2	3	8	2
Hematocrit	41	46	47	37	47	27	35	29	38	31	47
Sedimentation rate (mm./hr.)	8	0.7	0.5	0	5	53	1	12	18	2	2.5
Red blood cells	N	N	N	N	N	N	N	Hypochromic	Target cells polychrom.	Oval nucleated	Oval nucleated
Platelets	N	N	N	N	N	Slightly smaller	Slightly smaller	Smaller	Slightly smaller	*	*
White blood cells											
P.M.N.: (a) Stab (%)	2	--	1.2	3	8.6	11.8	1	5	5	21	0
(b) Seg. (%)	56	--	20.2	38	51.0	48.3	29	30	24	0.7	0
Eosinophils (%)	2	--	8	5	7.9	6.5	1	3	25	3.7	25
Basophils (%)	0.5	--	0	0	0.2	0.1	0	0	0	0.7	4
Lymphocytes (%)	34.5	--	63.5	53	23.4	29.4	55	58	45	55.3	60
Monocytes (%)	5.0	--	6.8	1	8.1	3.9	14	4	1	17.8	11

N = resemble normal human cells.
*"Thrombocytes" (see text).

and fast rate in opossum and cat. No correlation was apparent between fibrinogen level and sedimentation rate. (2) Low hematocrit values in cat, raccoon, and chickens. No recent phlebotomy had been carried out in these animals. (3) Red cells from the opossum showed polychromasia and suggested target cells. Duck and chicken erythrocytes were oval and nucleated. (4) Duck and chicken smears and wet preparations revealed "thrombocytes" rather than the cellular fragments called platelets in other species. Thrombocytes appeared as complete cells with nuclei about 3 times larger than human platelets, and were spheroid rather than discoid in shape. There was some difficulty in differentiating thrombocytes from small lymphocytes. However, careful morphologic and histochemical studies (Table II) made differentiation possible.

TABLE II. MORPHOLOGIC DIFFERENCES BETWEEN THROMBOCYTES AND SMALL LYMPHOCYTES IN CHICKEN BLOOD

	NUCLEUS	CYTOPLASM		TENDENCY TO CLUMP
Thrombocyte	Small, oval, concentric	Azure granules 5-6 light violet	Glycogen ++++	+
Small lymphocyte	Large, round, eccentric	± blue to dark blue	±	0

Coagulation studies are shown in Table III. Clotting times appear to be primarily dependent upon technique. Venepuncture in the monkeys and cardiac puncture in the opossum were technically difficult. Venepunctures in ducks and chickens were performed with ease, yet the silicone and glass clotting times were very similar. Clot retraction was present in all samples except those from the avian species. Clot lysis was not observed in any sample. Platelet counts varied considerably. Again, the duck and chicken were remarkable in showing no platelets, but doubly refractile spheres about 3 times larger than human platelets. Preparation of platelet suspension was unsatisfactory in these species as the thrombocytes appeared to sediment with the white cells. This was also true of the sedimentation of cow and sheep platelets.

Tests for prothrombin, proconvertin, and proaccelerin were the usual one-stage procedures which employ materials from several mammalian species. The thromboplastin used was Simplastin (a rabbit tissue thromboplastin); the substrates for assaying prothrombin and proconvertin were modified beef plasmas, and for assaying proaccelerin, aged human plasma. In these test systems prothrombin and proconvertin varied considerably from species to species, appearing remarkably low in the opossum, chicken, and duck. Among the mammals, man had by far the lowest proaccelerin level; chicken and duck levels were even lower.

Substrates for the assay of the 3 factors, antihemophilic, plasma thromboplastin component, and Hageman, are plasmas from human subjects congenitally devoid of these factors. Estimation of the concentration of these factors is based upon the ability of dilutions of the test plasma to restore the recalcification time and prothrombin consumption of the deficient plasma. High activity of AHF was observed in cow and sheep plasmas; chicken and duck

TABLE III. COAGULATION STUDIES EMPLOYING STANDARD TEST SYSTEMS[4, 5]
(Average of All Determinations (See Table I) for Each Species)

STUDY	MAN	MONKEY	COW	SHEEP	DOG	CAT	RABBIT	RACCOON	OPOSSUM	CHICKEN	DUCK
Clotting time 37° C.											
Glass (min.)	7.5	5	7	3	8.6	8	5	7	3	9	9
Silicone (min.)	22	8	12	3	17	17	15	25	3	10	10
Clot retraction	4+	4+	2+	3+	4+	4+	4+	4+	4+	0-tr.	0-tr.
Clot lysis	0	0	0	0	0	0	0	0	0	0	0
Platelet count ($\times 10^3$/mm.^{3})	293	721	212	415	296	394	750	500	375	150*	324*
Platelet ptpln. (%)†	100	88	27‡	10‡	80	86	200	350	100	‡	‡
Platelet acc. (%)§	100	82	<5‡	<15‡	130	136	200	100	50	‡	‡
Prothrombin (%)	100	185	20	15	139	49	95	165	0	4	15
Proconvertin (%)	100	193	42	50	1,140	129	315	325	0	0	0
Proaccelerin (%)	100	158	200	200	1,370	1,080	2,000	1,200	600	5	30
AHF (%)	100	120	830	1,000	46	200	300	80	650	50‡	35‡
PTC (%)	100	100	117	100	120	150	100	100	85	<5	<5
Hageman (%)	100	115	333	600	60	168‖	100	300	225	0	0
Fibrinogen (%)	100	117	245	140	210	45	143	120	140	79	100

*"Thrombocyte count."
†Platelet prothromboplastin.
‡See text.
§Platelet accelerin.
‖Excluding Cat 2.

TABLE IV. SPECIES SPECIFICITY OF TISSUE THROMBOPLASTINS
(Prothrombin Times in Seconds of the Various Plasmas, Using Thromboplastins as Listed)

THROMBOPLASTINS	HUMAN	MONKEY*	COW*	SHEEP*	DOG	CAT	RABBIT	RACCOON	OPOSSUM	CHICKEN	DUCK*
Human brain	13.8	13.0	22.5	12.2	7.5	13.2	13.8	9.4	43.2	149	360
Cow brain	43.0	26.0	18.8	13.5	14.2	36.4	25.2	20.8	42.4	105	450
Sheep brain	30.8	21.4	14.8	11.4	13.4	37.0	27.4	15.4	68.0	150	---
Dog brain	64.4	63	41.0	57.0	47.0	71.4	58	74.0	43.0	>600	---
Cat brain	23.8	16.4	11.8	13.4	10.8	19.8	19.6	12.4	47.4	142	---
Rabbit brain	12.4	12.4	17.8	14.2	8.2	11.8	8.2	8.8	31.4	91	203
Raccoon brain	29.8	39.8	32.2	21.8	12.8	24.4	20.4	13.4	44.8	137	---
Opossum brain	46.4	47.0	45.8	41.0	24.2	37.0	28.4	18.8	18.8	146	270
Chicken brain	37.4	44.0	83.0	33.1	21.8	24.8	24.0	27.0	42.0	11.4	14.0
Duck brain	90	71.5	119.0	55.0	31.8	>600	26.4	33.2	51.0	15.5	13.1
Beef lung	15.4	21.8	11.8	12.8	6.8	12.8	6.8	9.8	19.4	69	201
Soluplastin (horse)	14.8	19.8	25.4	14.0	8.2	11.2	7.0	9.8	24.8	53	160
0.85 per cent NaCl	143.8	130	86.0	68.0	68	104	63	51.0	>120	>600	370

*Frozen plasmas (pooled).

plasmas were able to restore prothrombin consumption, but only partially to shorten the recalcification time, thus exact comparison to mammalian plasma was not possible. The avian plasmas had no corrective effect on either the PTC or the Hageman-deficient plasma. In averaging the cat plasma levels of Hageman factor, one cat was omitted. On 3 separate occasions this cat showed a remarkable degree of Hageman factor deficiency (<5 per cent). Fibrinogen was assayed as the degree of turbidity resulting from heating the sample to 56° C.[5] No very remarkable differences were observed among the species studied.

1. *Species Specificity of Tissue Thromboplastins.*—Results are shown in Table IV. Thromboplastins from the brains of as many as possible of the various animal species were prepared. Fresh plasmas were used if possible; if not, frozen pooled citrated plasma was substituted. Duck, chicken, and opossum plasmas gave the shortest clotting time in the presence of the homologous brain thromboplastins. Among mammals other than the opossum such specificity was not apparent. Most plasmas clotted fastest with rabbit brain. On the other hand, the brains of two different dogs showed little thromboplastic activity. Opossum, duck, and chicken brains had little thromboplastic action on most mammalian plasmas.

2. *Species Specificity of Thrombin-Fibrinogen Reaction.*—In order to test the thrombin-fibrinogen reaction it was necessary to prepare these reagents from each species. Barium sulfate-adsorbed plasma was used as an impure fibrinogen. Thrombin was prepared as for prothrombin assay in Method 3 (see below). Table V shows the results. Opossum "thrombin" prepared by this method showed almost no activity. Opossum fibrinogen clotted very slowly with all thrombins, eventually forming a strong solid clot. Chicken fibrinogen clotted only with chicken thrombin. Only minor differences in specificity were noted among the mammals.

TABLE V. SPECIES SPECIFICITY OF THROMBIN-FIBRINOGEN REACTION*

	FIBRINOGEN						
THROMBIN	HUMAN	DOG	CAT	RABBIT	RACCOON	OPOSSUM	CHICKEN
Human	*12.8*	9.9	14.0	16.0	13.0	61.0	>300
Dog	16.0	*12.0*	15.0	20.0	15.0	232	>300
Cat	22.0	13.2	*15.1*	14.6	13.0	180	>300
Rabbit	18.0	13.5	17.0	*13.2*	12.2	72.5	>300
Raccoon	18.1	13.0	19.5	18.5	*14.8*	145	>300
Opossum	300	300	300	300	300	*45.3*	>300
Chicken	17.0	13.5	15.8	15.0	13.2	41	*21.0*

*Two-tenths milliliter of thrombin (each species) plus two-tenths milliliter of $BaSO_4$ plasma (each species). Clotting time in seconds.

3. *Prothrombin-Proconvertin Concentration.*—A comparison of the prothrombin-proconvertin concentrations determined in homologous systems was carried out.

Three milliliters of oxalated plasma from each species was mixed with 300 mg. $BaSO_4$, the $BaSO_4$ was centrifuged, washed, and eluted with 0.5 ml. 0.2 M sodium citrate. One-tenth milliliter of the eluate was then activated with 0.1 ml. homologous brain extract, 0.1 ml. homologous $BaSO_4$ plasma diluted 1:5,

0.5 imidazole buffer pH 7.3, 3.7 ml. saline and 0.5 ml. 0.15 M $CaCl_2$. At 1 minute intervals, 0.2 ml. samples were added to 0.2 ml. homologous $BaSO_4$ plasma. The minimum clotting time and activation time was recorded. Clotting time was converted to units by reference to prothrombin Method III curve.[4]

Table VI shows the prothrombin-proconvertin concentrations determined by this system. Concentrations found in most mammals were fairly similar but were extremely low in the opossum and chicken. Attempts to improve the yield of chicken thrombin included incubation for 1 hour and addition of a bovine proconvertin fraction. Neither procedure produced a significant increase.

TABLE VI. PROTHROMBIN-PROCONVERTIN CONCENTRATION EMPLOYING HOMOLOGOUS SYSTEMS

SPECIES	"UNITS"	ACTIVATION TIME (MIN.)
Human	260	3
Dog	144	7½
Cat	200	2
Rabbit	250	1
Raccoon	206	1
Opossum	21	10
Chicken	65	2

TABLE VII. THROMBOPLASTIN GENERATION TESTS

SPECIES	NUMBER OF DETERMINATIONS	PER CENT OF HUMAN (AVERAGE)
Man	11	100
Monkey	3	78
Cow	3	18
Sheep	1	25
Dog	3	70
Cat	4	50
Cat No. 2	3	<5
Rabbit	2	58
Raccoon	1	40
Opossum	1	110
Chicken	4	0
Duck	2	0

4. *Thromboplastin Generation Tests.*—Thromboplastin generation tests were carried out on most of the animals. Results shown in Table VII are for homologous systems in which equal volumes of $BaSO_4$-treated plasma (diluted 1:5) plus serum (diluted 1:10) plus washed platelet suspension (concentration adjusted to approximately that of whole blood) from each animal and 0.025 M $CaCl_2$ were mixed, and 0.1 ml. of this mixture together with 0.1 ml. 0.025 M $CaCl_2$ was added to human platelet-poor plasma. Relatively "normal" amounts of thromboplastic activity were generated in monkey, dog, cat (excluding No. 2), rabbit, raccoon, and opossum mixtures. Both sheep and cow plasmas assayed low and on additional testing the platelet suspensions were found to be relatively poor in platelets due to their sedimentation characteristics; hence, they were inactive as compared to human platelet suspensions. Cat No. 2 was remarkable in showing a consistent pattern compatible with Hageman factor deficiency, i.e., on 3 occasions generation of thromboplastic activity was markedly

deficient in the whole cat mixture, but if either human or normal cat $BaSO_4$ plasma or serum was substituted, thromboplastic activity improved greatly. Again chicken and duck were remarkable in generating no thromboplastin. Individual substitutions of $BaSO_4$ plasma, serum, and "platelets" from each in human systems, or substitution of human materials in the avian systems, produced no thromboplastin. If duck or chicken platelet-poor plasma was substituted for the human as a substrate, clotting time was very long. On the other hand, human thromboplastin generation mixture added to duck plasma as a substrate gave a prompt clot (14 seconds on duck plasma, 10 seconds on human plasma).

5. *Miscellaneous Observations.*—Platelet-poor plasma from the duck and chicken did not clot (clotting time $>$ 1 hour) on recalcification in glass tubes. If avian "platelets" (prepared by the same method used for human platelets) were added, no clotting occurred, indicating that the "platelet suspensions" probably contained no thrombocytes. When human platelets were added clotting did occur, although slowly.

Clotting times of whole avian blood, following good venepuncture, were not significantly shorter in glass than in silicone. This suggested the possibility that avian plasma was devoid of "glass factor." Chicken plasma and human plasma were "glass-activated" by exposure to a large glass surface (Pyrex wool). Each was tested for glass factor by adding 0.05 to 0.2 ml. human silicone tube-collected plasma in silicone tubes, and recalcifying the mixtures. The human glass-exposed plasma showed a marked clot accelerating effect on the silicone plasma, but the chicken glass-exposed plasma showed no accelerating but actually an inhibiting effect. Chicken plasma was tested for inhibitors to all human factors by incubating for 1 hour at 37° C. normal human plasma with (1) chicken plasma and (2) oxalated saline. The two mixtures were then tested for prothrombin, proconvertin, proaccelerin, plasma thromboplastin component, antihemophilic factor, and Hageman factor. No inhibiting effects could be found with the possible exception of the Hageman factor assay in which a slight prolongation of clotting time was observed.

DISCUSSION

This study emphasizes the fact that variations in coagulation factors among animals are great and that the test systems employed in animal experimentation should be carefully scrutinized. For example, it is obvious that the hemostatic mechanism in avian species must be very different from that in the human, but the interpretation of the duck's deficient thromboplastin generation test must be guarded, since it is also obvious that from the duck's point of view its own system is adequate in its function of preventing excessive bleeding following injury. The remarkable lack of sensitivity to glass activation and lack of Hageman factor activity in avian plasma has been noted by others.[9, 10] Our studies suggest that chickens and ducks also lack proconvertin (Factor VII and/or Stuart factor) and PTC activity. Unfortunately, we were unable to test for PTA activity.

Tests for species specificity of brain tissue as thromboplastins in the prothrombin time test showed there was no real specificity among the mammals

with the exception of the opossum. Chicken or duck brains showed great activity with chicken or duck plasmas and poor activity with mammalian plasmas. Rabbit brain, beef lung, and human brain were the most active thromboplastins in mammalian systems.

Again species specificity of the thrombin-fibrinogen reaction is not apparent among the mammals, but chicken plasma was clotted only by chicken thrombin.

Employing homologous systems the prothrombin-proconvertin concentrations among the mammalian group with the exception of the opossum did not vary greatly. The yield of thrombin from the dog mixture was somewhat low and the activation time long; this may reflect the poor thromboplastic activity of the dog brain suspension used. Both opossum and chicken showed low levels of thrombin yield from the activation mixtures.

Thromboplastin generation tests on each animal employed homologous generating mixtures but human plasma as a substrate in order to facilitate comparison. Among the mammals the cow and sheep mixtures generated low thromboplastic activity; this was found to be due to relatively inactive platelet suspensions, due in turn to the particular sedimenting characteristics of cow and sheep platelets. On the other hand, chicken and duck generating mixtures did not form any plasma thromboplastin even when tested on homologous substrates. This confirms Wartelle's recent report.[11] Human platelets, adsorbed plasma, and serum were individually substituted in the avian thromboplastin generation tests without effect.

One remarkable finding was the marked deficiency of Hageman factor in one (No. 2) of the 5 cats tested. Assay for Hageman factor on 3 separate occasions showed levels of less than 5 per cent as compared to normal human or normal cat. Thromboplastin generation was also very low in this cat but could be restored by substitution of either normal human or cat adsorbed plasma or serum. Unfortunately, this cat died before arrangements for mating could be made.

CONCLUSIONS

Routine hematologic and coagulation studies on 8 mammalian and 2 avian species showed certain remarkable differences. Fowl showed, instead of platelets, large thrombocytes, which were nucleated cells distinguishable from small lymphocytes by careful histologic technique and thought to resemble mammalian platelets because of their high glycogen content and tendency to clump.

Standard coagulation studies showed the following remarkable findings: (1) Negligible differences between clotting times in glass and silicone tubes in chicken and duck despite good venepuncture technique; (2) Negligible clot retraction in fowl blood; (3) Low levels of prothrombin and proconvertin in opossum, chicken, duck, sheep, and cow; (4) Levels of proaccelerin far higher than human in all mammals, and lower in fowl; (5) Very high levels of AHF in cow and sheep plasmas; (6) Rather uniform levels of PTC in all mammalian plasmas and complete lack of PTC in chicken and duck plasmas; (7) Levels of Hageman factor in all mammals, except one cat (< 5 per cent), which were

only moderately different from human. No Hageman factor activity was found in duck or chicken plasmas. "Glass factor" activity was also absent in duck and chicken plasmas.

Brain tissue thromboplastin showed a definite species specificity only in the opossum, chicken, and duck.

The thrombin-fibrinogen reaction in homologous systems showed that opossum fibrinogen was slowly clotted by all thrombins, but opossum thrombin clotted only opossum fibrinogen. On the other hand, chicken thrombin clotted all of the fibrinogens, but chicken fibrinogen was clotted only by chicken thrombin.

Employing homologous systems opossum and chicken showed very low concentrations of prothrombin-proconvertin.

Thromboplastin generation was very poor in one of 5 cats tested. This same cat had a low level of Hageman factor on 3 occasions and was considered to be deficient in Hageman factor. Thromboplastin generation of chicken and duck, even when using homologous substrate, was negligible.

The importance of using homologous species systems in the study of blood coagulation mechanisms is emphasized.

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HUMAN LIVER GLYCOGEN LEVELS

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ALTHOUGH many aspects of carbohydrate metabolism in man have been studied intensively, relatively few quantitative data are available on the glycogen content of the human liver during life. Since species variations in hepatic glycogen content are large, the voluminous data accumulated from animal experiments cannot be applied to human beings. Determination of the normal range of human liver glycogen, and of variations to be expected under different conditions, is of practical as well as theoretical importance. Definition of the upper limit of the normal range would be particularly valuable. Excessive hepatic glycogen deposition is observed in glycogen storage disease and in some patients with diabetes.[1-4] The glycogen levels reported in such cases do not exceed those seen in normal animals of some species after carbohydrate loading,[5] but probably exceed the upper limit of the normal human range.

This report adds 37 determinations of human liver glycogen to the literature and summarizes some of the published data from similar studies. A tentative normal range is suggested. An additional case of excessive glycogen storage in diabetes is reported.

MATERIALS AND METHODS

Surgical biopsy of the liver was performed immediately after entering the abdomen in the course of diagnostic laparotomy or intraabdominal surgical procedures. Biopsies were always obtained from grossly normal portions of the liver, in patients who had hepatic metastases. Most specimens were obtained from the right lobe. Glycogen was determined by the method of Good, Kramer, and Somogyi,[6] using the Nelson[7] method for the determination of glucose after hydrolysis. Liver glycogen was reprecipitated once before hydrolyzing. Duplicate glycogen determinations were performed in all cases. The values reported below are the averages of these duplicates. In several cases, the duplicate determinations were not in the same range; these cases were discarded for the series.

Preoperative preparation and anesthesia was relatively uniform in this series. Patients were fasted approximately 14 hours prior to surgery. The anesthetic agent was usually cyclopropane, often supplemented by intravenous succinylcholine. Preanesthetic medication included Demerol or morphine, and atropine or scopolamine. Patients were usually receiving slow intravenous infusions, often containing glucose, at the time biopsies were obtained. However, only small amounts, containing less than 10 Gm. of glucose, had been administered before the liver biopsy.

Patients' diet during the preoperative periods provided 1,400 to 1,900 calories per day, with 125 to 200 Gm. of carbohydrate, 50 to 70 Gm. of protein, and 70 to 80 Gm. of fat. However, the feedings offered were not always ingested completely. The long-range nutritional status of patients was estimated by comparing the patients' weights with the average for the appropriate sex, height, and age, obtained from standard tables.

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TABLE I. CLINICAL DATA AND LIVER GLYCOGEN LEVELS

SEX	AGE	WEIGHT*	DIAGNOSIS	LIVER GLYCOGEN (PER CENT)
		A. No Jaundice		
F	65	Overweight	Gastric polyp	3.41
M	59	Normal	Polyp of colon	1.70
M	80	Underweight	Gastric ulcer	2.92
M	42	Normal	Duodenal ulcer	0.90
M	42	Normal	Duodenal ulcer	2.41
F	67	Normal	Cholelithiasis	2.80
M	80	Underweight	Carcinoma of stomach	0.31
M	70	Underweight	Carcinoma of stomach	0.64
M	61	Normal	Carcinoma of stomach	1.04
M	63	Underweight	Carcinoma of stomach	1.26
F	73	Underweight	Carcinoma of stomach	1.70
M	76	Underweight	Carcinoma of stomach	2.03
M	65	Underweight	Carcinoma of stomach	2.17
F	46	Underweight	Carcinoma of stomach	2.56
M	66	Underweight	Carcinoma of stomach	3.00
F	73	Underweight	Carcinoma of stomach	3.25
F	67	Normal	Carcinoma of stomach	4.00
M	66	Underweight	Carcinoma of stomach	5.78
M	76	Underweight	Carcinoma of stomach	5.98
F	63	Underweight	Lymphosarcoma of stomach	0.52
M	65	Underweight	Lymphosarcoma of stomach	1.61
F	49	Overweight	Carcinoma of gall bladder	0.56
M	72	Underweight	Carcinoma of colon	3.83
F	70	Underweight	Carcinoma of rectum	0.34
M	31	Normal	Carcinoma of rectum	1.56
F	55	Normal	Carcinoma of rectum	2.81
M	74	Normal	Carcinoma of rectum	3.04
M	68	Normal	Carcinoma of rectum	2.92
F	65	Underweight	Carcinoma of rectum	4.12
F	62	Overweight	Carcinoma of cervix	2.05
		Mean and standard error	2.22 ± 0.27	
		B. Jaundice Present		
F	52	Normal	Cirrhosis of liver	2.36
F	73	Normal	Cholelithiasis	1.99
M	54	Underweight	Carcinoma of pancreas	1.46
M	67	Normal	Carcinoma of pancreas	3.59
F	51	Underweight	Carcinoma of gall bladder	2.83
M	72	Normal	Carcinoma of stomach	0.47
		Mean and standard error	2.12 ± 0.46	
		Combined series		
		Mean	2.20	
		Standard deviation	1.39	
		Standard error of mean	0.24	

*Deviation of more than 15 per cent from the average weight for patients' height, age, and sex is indicated by "overweight" or "underweight." Weight tables of the Association of Life Insurance Directors and Actuarial Society of America were used (reproduced in Duncan, G. G.: Diseases of Metabolism, ed. 3, Boston, 1952, W. B. Saunders Company).

RESULTS

Table I presents the results of liver glycogen determinations in 36 patients. The patient with diabetes is excluded. The mean value for 30 patients without jaundice, 2.22 per cent, did not differ significantly from the mean for the 6 jaundiced patients, 2.12 per cent. For the combined group of 36 patients, the mean liver glycogen was 2.20 per cent, the standard deviation was 1.39 per cent, and the standard error of the mean was 0.24 per cent. In reviewing the individual data, correlations between liver glycogen values and diagnosis,

nutritional status (as represented by body weight), and age were sought. No correlations with any of these were found. An attempt to correlate liver glycogen levels with food intake during the 72 hours prior to surgery was unsuccessful, since we could not determine the actual intake during the preoperative period.

CASE REPORT

The patient was a 64-year-old white woman with a history of diabetes of 13 years' duration. She was admitted with complaints of anorexia, cough, and vomiting of 6 weeks' duration, and was found to have an abdominal mass. She was slightly obese, had arteriosclerotic heart disease with hypertension, failing vision, albuminuria, and ankle edema. Her diabetes was poorly controlled prior to exploratory laparotomy, with morning blood glucose values usually over 450 mg. per cent, and constant 3 to 4 plus glycosuria, on insulin dosage of 40 to 70 units of regular insulin per day. There was one episode of hypoglycemia, 32 hours prior to surgery, after the insulin dose was raised to 105 units on one day. She responded promptly to orange juice on this occasion, and continued to show glycosuria and hyperglycemia up to the time of surgery. Because there was no evidence of ketosis, and surgery was deemed imperative, laparotomy was performed in spite of the unsatisfactory status of diabetic regulation. Her liver glycogen at operation was 12.9 per cent. She went into severe acidosis postoperatively, was finally brought under control, then died suddenly of myocardial infarction. Postmortem liver glycogen was 9.0 per cent. The liver weighed 1,500 grams and was otherwise normal. Pancreatic islet tissue was essentially normal, as were the adrenals. The kidneys showed arteriolar sclerosis, as well as typical lesions of intercapillary glomerular sclerosis.

DISCUSSION

The liver glycogen values obtained in this study are similar to those reported by MacIntyre and associates,[8] Young and co-workers,[9] and Hildes and associates.[10] Analysis of variance, using the F test, failed to indicate heterogeneity among the 4 groups. The specimens in the two former studies, like ours, were obtained at laparotomy. However, the anesthetic preparation differed; MacIntyre obtained specimens under spinal anesthesia, and Young under Pentothal and ether. Hildes, on the other hand, obtained specimens by needle aspiration biopsy in unanesthetized patients. We had anticipated that there would be differences in the liver glycogen values among these series attributable to the anesthetic preparation. It is well known that, in experimental animals, agents such as morphine and ether produce hyperglycemia and glycogenolysis, through stimulation of adrenal epinephrine release. Failure to find such differences suggests that this mechanism may be less important in the human, or that other differences among the patients studied overshadowed the possible effects of epinephrine release. The latter is probably an important factor; the wide range of glycogen values found in all 4 series indicates considerable inhomogeneity among the patients in each one. However, the observations of Hildes, Sherlock, and Walshe[11] suggest that man is indeed less responsive to the hepatic glycogenolytic action of epinephrine than are some animal species.

Fig. 1 presents the combined data from this series and those of MacIntyre, Young, and Hildes and their respective co-workers. Three determinations by Bondy and associates,[2] based on needle aspiration biopsy after an overnight fast, are also included. The patients in the operative series were fasted 10 to 14 hours prior to surgery. The duration of fast in Hildes' series is not stated;

presumably it was also an overnight fast of about 10 to 14 hours. The median value for liver glycogen in the combined series is 2.16 per cent, and the mean is 2.35 per cent. The upper limit of the normal range, under these conditions of moderate fasting, appears to be about 6 per cent.

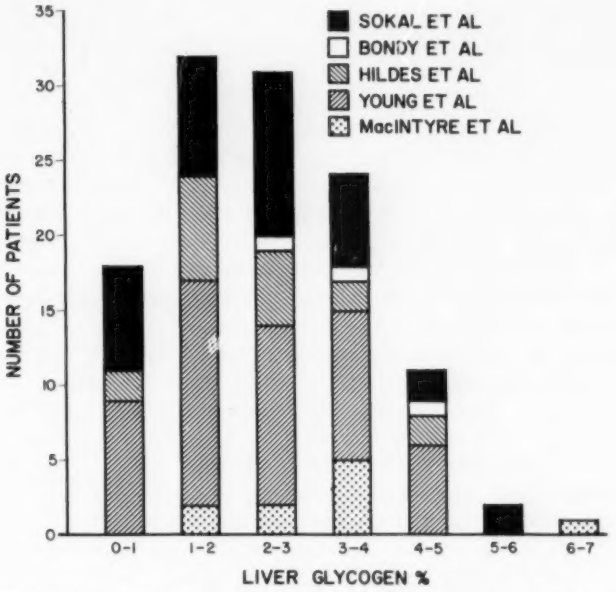

Fig. 1.—Distribution of liver glycogen levels after overnight fast. Data from present series and others are combined.

Neither in our series nor in those of MacIntyre, Young, and Hildes was there any correlation between liver glycogen levels and age or diagnosis. This is not surprising. No changes in carbohydrate metabolism specific for the age groups or diagnoses represented by these patients have been established. The liver glycogen levels in our 6 jaundiced patients did not differ from those in the remainder of the series. These patients did not have severe hepatocellular damage, and showed no clinical evidence of defective carbohydrate metabolism.

We had expected to find a correlation between nutritional status and the amount of liver glycogen. However, liver glycogen has a relatively rapid turnover rate.[12-14] Its concentration must therefore be determind principally by the metabolic and nutritional events of the 24- to 72-hour period preceding sampling, rather than by the long-range factors reflected by patients' weights. The wide range of values listed in Table I suggests considerable variation in these preoperative events among our patients. Although we were unable to determine these variations for individual patients, we know that there were in fact differences among them in food intake, caloric expenditure, and other metabolic factors during the preoperative period. Such variations were presumably also present in the other reported studies.

MacIntyre and Young determined the liver glycogen of patients given large glucose feedings during the immediate preoperative period, and reported values ranging from 3.2 to 9.7 per cent. Comparison of these values with those in Fig. 1 suggests that the human being can deposit 3 to 4 per cent of liver glycogen during a period of 10 hours or less. Bondy, who performed serial biopsies before and after breakfast in two patients, observed rises of 1.5 and 2.3 per cent over this much shorter time period. Experiments with glucagon suggest that human liver glycogen can be broken down rapidly.[15, 16] Thus, the *rate* of glycogen metabolism in the human probably does not differ very greatly from that in the albino rat, which deposits about 1 per cent of glycogen per hour after a large glucose feeding and can release about 3 per cent in one hour during maximal glucagon stimulation.[17] However, the *concentration* of liver glycogen in the human being exceeds significantly that observed in the rat under comparable conditions. The same is true of muscle glycogen.[10] Monkeys, on the other hand, probably have liver glycogen levels comparable to man[18]; while rabbits and dogs frequently exceed the normal human range suggested by the data reviewed here.

A distribution of liver glycogen values almost as broad as that shown in Fig. 1 is observed in laboratory animals when experimental conditions are not very rigidly controlled. We have observed considerable variation in liver glycogen levels of mice, rats, and rabbits even when they are maintained under uniform laboratory conditions and sampled at the same time. When, however, animals are fasted long enough to reduce liver glycogen to very low levels, and then are given standard carbohydrate loads by stomach tube or by parenteral injection, the resultant glycogen levels fall within a much narrower range. Such conditions would be impracticable to impose upon patients, since fasts of at least 24 hours would be required. It will be necessary, therefore, to obtain data on fairly large numbers of patients in order to document differences of liver glycogen within the ranges for fasting or fed patients discussed above. On the other hand, individual determinations which fall well outside these ranges can probably be accepted as evidence of disturbed carbohydrate metabolism.

Although most patients with diabetes have low to normal levels of liver glycogen, as do depancreatized animals, an occasional patient has an unusually high level.[1-4] These patients may represent a special category of clinical diabetes and may be resistant to glucagon.[4] It is interesting that the excessive glycogen storage in our patient was demonstrable even in the postmortem specimen. Unfortunately, studies with glucagon were not performed in this patient.

Liver glycogen values in the hepatic form of glycogen storage diseases are often as high as 12 to 16 per cent.[19] Such values are clearly outside the normal human range, even after high carbohydrate feeding. However, values in the range of 6 to 10 per cent are frequently found. These are above the fasting range indicated by the data reviewed in this paper, but are within the normal range following glucose feeding. Even lower values have been reported in well-documented cases of glycogen storage disease.[20-22] Thus, there is considerable overlap between glycogen levels in this disease and the normal range. The

diagnosis of hepatic glycogen storage disease should, therefore, be based principally on the characteristic metabolic and biochemical defects, rather than on the liver glycogen concentration.

SUMMARY

Thirty-six determinations of human liver glycogen during life are recorded, and an additional case of excessive liver glycogen deposition in diabetes is reported. The data presented agree with other operative series reported, and with results obtained through needle aspiration biopsy in unanesthetized patients. Review of the combined data indicates that the upper limit of the normal range for human liver glycogen after an overnight fast is about 6 per cent, and that concentrations of 3 to 10 per cent are seen after large glucose feedings.

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A SOLITARY ERYTHROBLASTOMA OF THE LIVER

Report of Case

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INTRODUCTION

A REVIEW of the literature indicates that the occurrence of a solitary erythroblastomatous tumor is sufficiently rare as to be unique. As of this date, we have been able to find only 11 recorded cases of erythroblastic tumors[1-9] in which the erythroblastic element was a predominant feature in both clinical and pathologic findings in the patient. Unfortunately, the problem has been greatly clouded by semantics. It is almost impossible to be certain from the literature as to the precise kind of tumor present. The first report of erythroblastic myeloma was published by Ribbert[1] in 1904, and since then the above group has been found acceptable as cases in which there was a well-defined erythroblastomatous constituent. The erythroblastic feature was prominent in all of them and was of multiple origins, or at least had multiple metastatic areas. It is well known that this group of tumors is connected with the myeloma group or is included as a part of the myeloma group. On the other hand, the relationship of the erythroblastic group with the hemoblastic sarcomas is found in a single case report. The finding of an erythroblastic element in the tumors of the myeloid system in species other than man is recognized; it seems to be an astonishingly rare occurrence in human beings. For the above reasons, we find it necessary to report a well-defined case of solitary erythroblastic tumor of the liver which occurred in a 79-year-old white man in this hospital.

CASE REPORT

Clinical Data.—The patient was a 79-year-old white man admitted to this hospital for the first time on March 24, 1958. He complained of pain in the right upper quadrant, which radiated transversely across the abdomen. He also stated that he had generalized weakness. The onset was said to have been on the morning of March 17, 1958, when he awakened but could not get out of bed because of dizziness. When he finally did get up, he fell to the floor, "Like someone hit me with an iron bar." He had pain in the right upper quadrant with soreness in this area immediately thereafter. He had had 3 admissions to the Veterans Administration Hospital in Wadsworth, Kansas, the first for right inguinal hernia repair, the second for a transurethral resection for median bar hypertrophy, and the third for high

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left saphenous ligation for varicose veins. He had been a moderate drinker but used wine only. He had been a farmer and railroad section hand all of his life. He reported that his urine was dark and that he urinated with a slow stream.

Physical examination revealed a well-developed, fairly well-nourished, white man, admitted via wheel chair. He was 5 feet, 9 inches tall and weighed 158 pounds, having lost some 12 pounds during the preceding month. The temperature, pulse, and respirations were normal, and blood pressure was 110/70 mm. Hg. The salient features of the general physical examination were: (1) pendulous breasts, (2) weight loss, as evidenced by rather flabby abdominal and chest walls and poor tissue turgor, (3) an enlarged and tender liver, 4 finger breadths below the costal margin, (4) questionable tumor mass in the gall bladder area, and (5) chronic osteoarthritis.

Urinalysis revealed a 1+ albuminuria. The VDRL test was negative. The hemogram was normal. Blood urea nitrogen 16 mg. per cent; fasting blood sugar 81 mg. per cent; alkaline phosphatase 0.8 Bodansky unit; thymol turbidity 8 Maclagan units; total protein 4.6 per cent with an A/G ratio of 2.9 to 1.5, and a serum amylase of 60 units. Icterus index was 10 units; cholesterol esters 60 mg. per cent. Urine smears and cultures were negative on two occasions. The Bromsulphalein test revealed 44 per cent retention at 45 minutes. Prothrombin time was 15 seconds with a control of 15 seconds. The sodium was 142 mEq.; potassium 3.8 mEq.; CO_2 combining power 55 volumes per cent, or 25 mEq.; calcium 9.3 mg. per cent; and phosphorus 3.1 mg. per cent. The cephalin flocculation was 2+ at 24 hours and 3+ at 48 hours. The bleeding and coagulation times were normal.

An ECG made on the day of admission was normal. An x-ray of the chest on March 24, 1958, showed elevation of both leaves of the diaphragm with resultant decreased aeration in the lower lung fields. This was felt to be due perhaps to abdominal pain, the possibility of peritonitis being suggested. A gall bladder series revealed nonvisualization of the gall bladder. An upper gastrointestinal series indicated rather persistent deformity of the gastric antrum. There were possible retroperitoneal lymph nodes, manifested by forward displacement and deformity of the posterior wall of the second part of the duodenum. Probable hepatomegaly was also noted. A flat plate of the abdomen, taken April 15, revealed questionable evidence of enlargement of the gall bladder.

Treatment consisted of a low-fat diet; Diuril 0.5 Gm. b.i.d.; Darvon, APC, and Isuprel linguettes 10 mg. p.r.n. for syncopal attacks; and ephedrine grains 3, and amytal grains ¾ for the same attacks, which developed sometime following admission to the hospital.

On April 13, 1958, following breakfast, the patient exhibited slight cyanosis. During the morning he suddenly lost consciousness, had an involuntary bowel and bladder action and momentary absence of pulse. A Stokes-Adams attack was considered to be the most likely cause for the episode, and therefore the medications mentioned above were given. On April 15, 1958, a note was made that the patient appeared to be terminal. There was auricular fibrillation accompanied by frequent vomiting of coffee-ground material. The heart rate varied between 70 and 140; there was an irregular irregularity. The blood pressure was 90/60 mm. Hg. On April 17, the general condition of the patient was failing fast. Again there was emesis of coffee-ground material. The pulse was hardly perceptible. The blood pressure could not be obtained. On April 18, 1958, there was no pulse, and the respirations were very shallow. There was involuntary voiding and passage of black tarry stools. The patient died at noon that day.

Autopsy Findings.—The autopsy was done following arterial embalming. External examination was grossly normal. There was moderate rigidity, lividity was minimal and dependent. The body was well developed and rather well nourished. Although the body could not be considered obese, subcutaneous fat was abundant. The muscles were of good tone and well developed. On opening the peritoneal cavity a large cystic mass which measured 15 by 15 by 15 cm. was discovered in the right lobe of the liver. An attempt to remove the mass caused it to rupture and spill a large quantity of dark red-brown material which had a thick grumous consistency, and it was flecked throughout with gray-white flocculants which measured 1 to 3 mm. The material had a rather strong odor. The

Fig. 1.—This picture shows blocks taken from the edge of the tumor where it encroaches upon the liver. The entire center of the tumor was necrotic and did not fix. The light triangular area on the right-hand specimen is normal liver. Note the ragged and hemorrhagic-looking appearance of the tumor which remains.

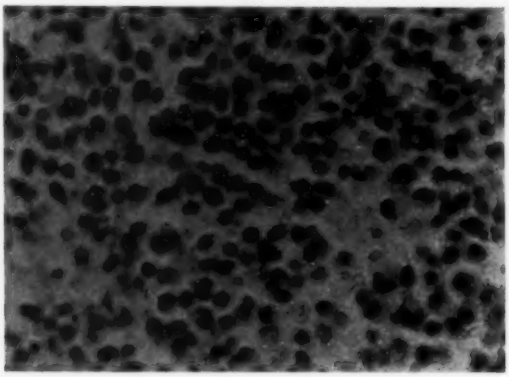

Fig. 2.—The megakaryocytes are readily seen, and the majority of the other cells present are in the erythroid element group and include many nucleated red cells.

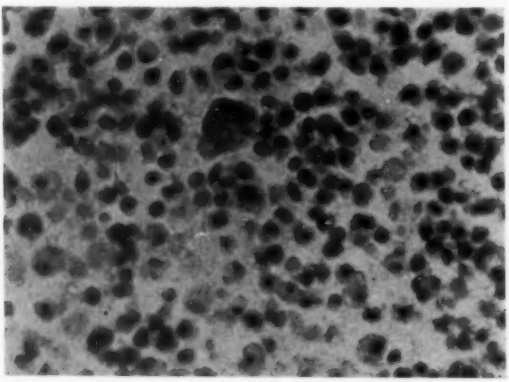

Fig. 3.—A well-defined megakaryocyte is above the center of the field. These are fairly abundant throughout the tumor. Note the nucleated red cells.

pleural cavities contained dark-brown fluid, the left approximately 500 ml. and the right 250 to 350 ml. The left lung weighed 600 grams, the right lung weighed 500 grams; they were grossly normal except for old calcified nodules in the upper lobe of the right lung. The heart weighed 375 grams; both epicardium and endocardium were smooth; the musculature was firm and of good texture. The coronary arteries were not remarkable. The aorta was normal with rather a minimal amount of atheromatosis. The spleen weighed 300 grams; it had a thickened capsule and was firm and rubbery. On cut section the pulp was dark purple in color; it remained firm and did not show shrinking or bulging. Some portions were somewhat darker purple than the remainder, this was probably an embalming artefact. The pancreas weighed 100 grams, on cut section it was grossly normal. The liver was greatly enlarged due to the mass which occupied the right lobe. The tumor occupied the right lobe, measured approximately 15 cm. across, was ball-shaped and had a tremendously thick and fibrosed wall. It occupied almost the entire right lobe of the liver and on section much of the internal portion was ragged and necrotic (Fig. 1). The right adrenal had been compressed by the liver mass. The left adrenal was autolyzed. The esophagus, stomach, and intestines were normal, except the colon which was attached to the mass. The left kidney weighed 150 grams, the right kidney weighed 200 grams; they were grossly normal except for a number of solitary retention cysts scattered throughout their cortices. The urinary bladder was normal.

Microscopic Data.—

Lung: The lung shows congested capillaries. The alveolar walls are thin, and there is no consolidation or excess fluid in the alveolar spaces.

Heart: The fibers are normal in appearance except for a few focal areas where the ends of the fibers are frayed. There are also some focal spots of fibrosis which are small.

Spleen: Microscopic section shows the pulp to be somewhat edematous. Malpighian corpuscles are clearly defined in some areas. The balance between red and white cells is normal, the capsule is fibrosed.

Pancreas: There is a slight increase in fibrous connective tissue scattered throughout the pancreas. The islets are within the range of normal construction.

Liver: Sections of liver away from the tumor show the separated liver cord cells with excess pigmentation often found in patients of this age group. Many sections of the tumor show the following characteristics: (1) A vascular tumor around the edges of which there are compressed liver cord cells. (2) The tumor cells are of the myeloid group, obviously erythroblastic in their origins. Megakaryocytes are found, as well as all stages of nucleated red cells and primitive types of red cells up to the stage of almost normal reticulocytes. No normal-looking red cells are found in the tumor proper (Figs. 2 and 3). (3) The tumor grows in groups and strands and on frondlike arrangements and has some areas which are quite similar to the erythropoietic areas in normal bone marrow. The great majority of cells in this tumor are primitive, undifferentiated, erythroblastic-type cells with large, dark, blue-staining nuclei and scant pink-staining cytoplasm. (4) Special stains done for hemoglobin on this tumor show that these cells have the capability of producing hemoglobin in small amounts in the cytoplasm of the more mature erythroblasts. (5) There are large areas of necrosis. (6) Although it is not a common feature, there are some areas in which the perivascular arrangement is in pseudorosettes of a rather poor quality.

Gastrointestinal tract: Microscopic sections are normal.

Adrenal: Sections were normal.

Kidneys: Sections taken away from the retention cysts show cloudy swelling of the tubules and normal glomerular structures.

Bone marrow: Multiple sections of the bone marrow appear to be somewhat hyperplastic in some areas, but in other sections are completely normal. Bone marrow was studied from the sternum and the body of the vertebrae.

DISCUSSION

Although the problematic nature of the myeloma group of tumors remains obscure, the microscopic feature of this tumor places it in the erythroblastic group. There is no question concerning the cytologic characteristics found, nor of the general arrangement of the neoplastic tissue. It is surprising that the more primitive tumor cells appear malignant but evidently did not metastasize from their site of origin. As to the precise classification of this tumor, it should probably be placed in the general descriptive literature under the term of hemangioblastomatous myeloma, solitary. However, this seems to us to be a complicated and unnecessarily difficult designation, and for that reason the words "erythroblastoma, solitary" have been used to designate it. The special stains done and the studies on the section leave no question concerning identification of the tumor cell type. It is in the erythroid-myeloid series and shows a good balance between the early forms of erythroblastomatous cells and the more adult forms of nucleated red cells. The curious thing noted is that while the cells mature to the level of nucleated red cells they do not make any visible effort to go beyond this to the reticulated and nonnucleated red cell form and seem to have no capacity to escape into the general circulation. For this reason the patient did not have polycythemia vera. Whether or not this may be a myeloma in which the balance between the so-called true myeloma cells and the small lymphocytes, or even small erythroblasts, has taken over is not clear. The erythroid multiple myeloma was discussed by Taylor[2] in 1947. In our study we were able to show that there is a slight amount of hemoglobin in these cells, but really very little, this is undoubtedly attributed to the fact of their immature state.

One of the most distinctive features in the present tumor is the angiomatous type of large, almost cavernous, sinus arrangements found near the edge of the tumor tissue. This tumor brings up the old problem of colonization of such a tumor from bone marrow myeloma or from autochthonous liver erythroblastomatous cells. The present case is contributory to the thinking in this area because it is obvious that this man's normal bone marrow is at least a bone marrow which is not a polycythemia marrow or one with malignant cells in it. It could not have contributed to colonization of the liver. Therefore, it must be assumed, in the present case, that the erythroblastoma of the liver arose primarily in the liver site in which it was discovered.

The case is also contributory because it brings up the problem of how the stimulus which evoked the erythroblastoma could be confined solely to the liver and not have produced the same type of lesion elsewhere in the bone marrow or myelopoietic system.

SUMMARY

Our review of the literature indicates that the present case is the first report of solitary erythroblastoma. In this case a reasonably normal balance was held between the megakaryocytes and nucleated red cell group. However, at this level of cell maturation the normal arrangement ceases, and there is

presented a great predominance of nucleated red cells without further maturation of the red cell series. The case shows some of the difficulties which may be expected to be encountered in reaching a premortem diagnosis in the myeloma group.

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THE ABSORPTION AND EXCRETION OF KANAMYCIN IN HUMAN BEINGS

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SINCE its discovery in 1956, kanamycin has been subjected to extensive laboratory and clinical studies.[2-6] These have demonstrated that this antibiotic is readily absorbed from parenteral sites but only slightly absorbed from the gastrointestinal tract.

Laboratory data from experiments with kanamycin will be presented in this paper. Included are: the maintenance of kanamycin serum levels following various dosage programs, the excretion rate of kanamycin in the urine, the effect of activity and fluid intake on the absorption and excretion of kanamycin, and the side reactions resulting from short-term kanamycin administration.

METHODS AND MATERIALS

All kanamycin absorption and excretion studies in this laboratory were performed on healthy adults of both sexes. The average age of the volunteers was 25 years with a range of from 18 to 52 years. Kanamycin sulfate was used in two solution strengths; the first contained 1,000 mg. in 3 ml. of diluent and the second (which was used in the majority of the studies) contained 500 mg. in 2 ml. of diluent (lot No. X502-58K89). All injections were made in the gluteal muscle using 1½ in. 20 gauge needles. In all experiments, pretest serum samples were obtained to rule out the presence of antibacterial substances. Kanamycin assays were performed using streptomycin assay agar with yeast extract and *Bacillus subtilis* A.T.C.C. 6633 as the test organism.* All urine excreted by the volunteers was examined for formed elements and albumin.

RESULTS

In the first experiment, kanamycin sulfate (0.25, 0.5, and 1.0 Gm.) was administered to 3 groups, each composed of 10 volunteers. Blood samples were drawn at 0, 1, 3, 8, and 24 hours and urine was collected in time periods of from 0 to 3, 3 to 8, 8 to 16, and 16 to 24 hours. The average serum levels of kanamycin following the above doses are presented in Fig. 1. While the kanamycin doses were in geometric relationship to each other (e.g., 1, 2, and 4), the average blood levels did not mirror the amount of antibiotic given. The augmentation of serum levels from the lowest dose to the highest at one hour was 177 per cent, at 3 hours, 185 per cent, and at 8 hours, 335 per cent; this

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suggests that absorption from parenteral sites may be slower when higher antibiotic concentrations are injected. The rate and amount of kanamycin excreted in the urine following the above doses are presented in Fig. 2. Approximately 90 per cent of the antibiotic test dose was identified in the urine during the first 24 hours after administration. Ninety per cent of the total amount excreted during the 24 hours was recovered in the periods of 0 to 3 and 3 to 8 hours. This urinary excretion pattern during the first 8 hours appears to be related directly to rapid decrease in serum concentration.

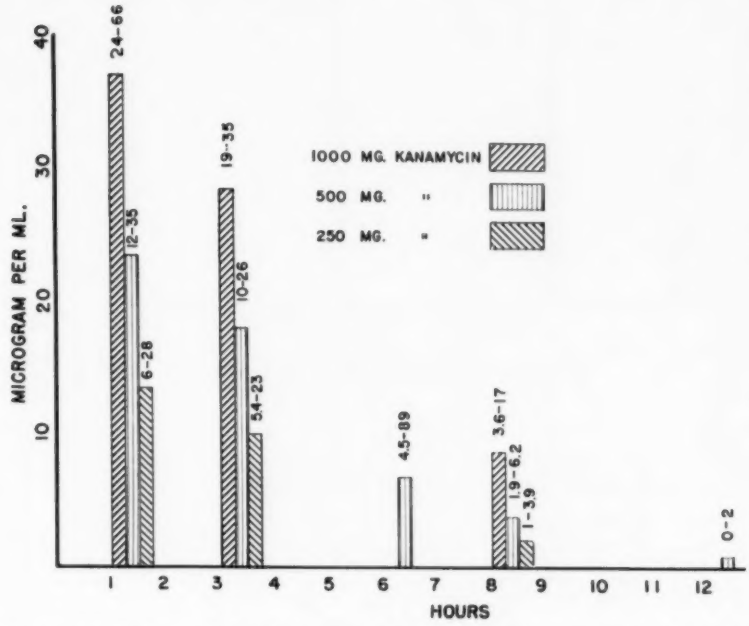

Fig. 1.—Kanamycin serum levels following the intramuscular injection of 0.25, 0.5, and 1.0 Gm. of kanamycin sulfate.

After the single-dose patterns of kanamycin serum concentrations were established, experiments were performed to study multiple dose serum concentration patterns. In the first of these, 15 volunteers were given 0.5 Gm. of kanamycin sulfate every 12 hours for a period of 72 hours. Blood samples were drawn for assay at 0, 1, 5, 8, 12, 13, 24, 25, 36, 37, 48, 49, 60, 61, and 72 hours to establish the average high serum concentration at one hour after injection and the average low serum concentration at 12 hours after injection; the results are presented in Fig. 3. The average high concentration ranged between 25 and 30 μg per milliliter with little or no tendency to accumulate and the average low was 0.9 μg per milliliter.

In the second of these experiments, 0.5 Gm. of kanamycin sulfate was administered every 8 hours to 5 volunteers. Blood samples were drawn at 0, 1,

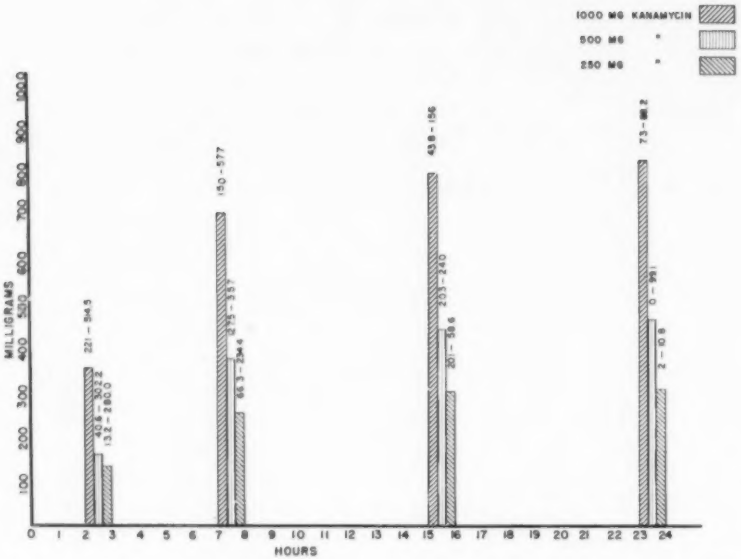

Fig. 2.—Average urinary excretion of kanamycin following the intramuscular injection of 0.25, 0.5, and 1.0 Gm. of kanamycin sulfate.

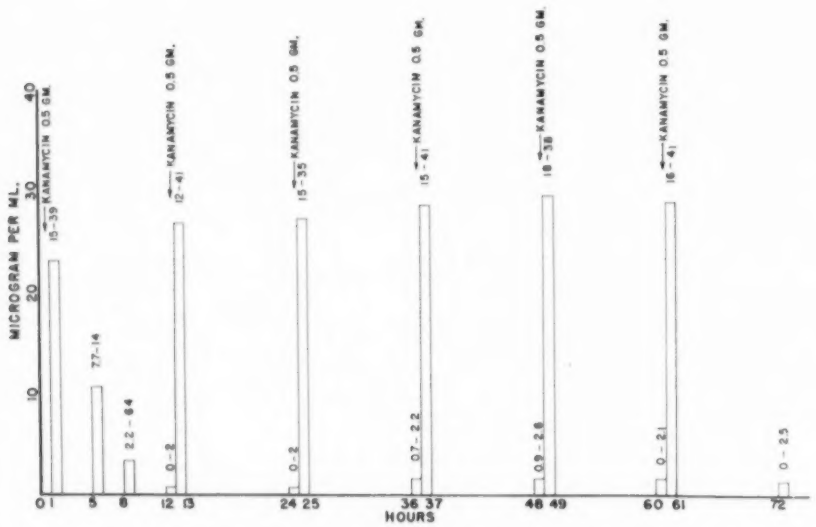
MICROGRAM PER ML.
HOURS
15 - 39 KANAMYCIN 0.5 GM.
7.7 - 14
2.2 - 6.4
0 - 2
(2 - 4) KANAMYCIN 0.5 GM.
0 - 2
15 - 35 KANAMYCIN 0.5 GM.
0.7 - 2.2
15 - 41 KANAMYCIN 0.5 GM.
0.9 - 2.8
18 - 38 KANAMYCIN 0.5 GM.
0 - 2.1
16 - 41 KANAMYCIN 0.5 GM.
0 - 2.5

Fig. 3.—Kanamycin serum concentrations following the intramuscular injection of 0.5 Gm. of kanamycin sulfate every 12 hours to 15 subjects.

8, 9, 16, 17, and 24 hours and assayed for kanamycin activity; the serum levels are presented in Fig. 4. The average high serum concentration measured at one hour after injection was 30 μg per milliliter and the average low concentration at 8 hours after injection was 4 to 5 μg per milliliter.

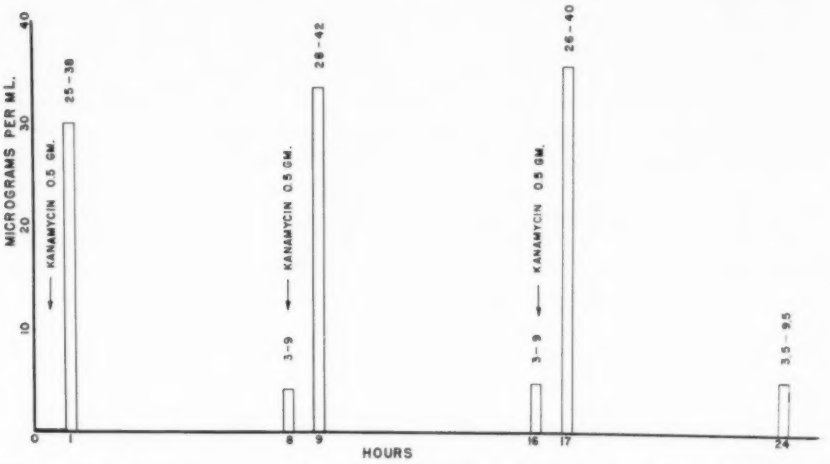

Fig. 4.—Kanamycin serum concentrations following the intramuscular injection of 0.5 Gm. of kanamycin sulfate every 8 hours to 5 subjects.

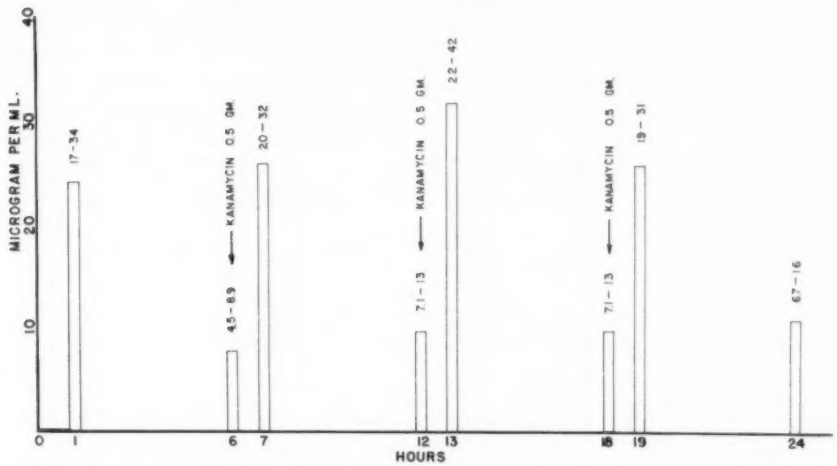

Fig. 5.—Kanamycin serum concentrations following the intramuscular injection of 0.5 Gm. of kanamycin sulfate every 6 hours to 5 subjects.

In the third experiment, 0.5 Gm. of kanamycin sulfate was administered every 6 hours to 5 human volunteers. Blood samples were drawn for assay at 0, 1, 6, 7, 12, 13, 18, 19, and 24 hours; the serum levels are presented in Fig. 5.

The average high serum concentration at one hour after injection was approximately 30 μg per milliliter and the average low concentration at 6 hours after injection was 8 to 10 μg per milliliter.

There was no essential increase in high kanamycin serum concentrations even though the serum contained 5 to 10 μg per milliliter of kanamycin at the time of the repeat injection in the latter two experiments in which kanamycin was injected every 6 or 8 hours. It is suggested that normally functioning kidneys have the ability to handle large amounts of kanamycin and that the higher the serum concentration of antibiotic is, the faster will be the rate of kidney excretion. To maintain antibiotic serum levels above 5 μg per milliliter, kanamycin should be administered by intramuscular injection every 6 to 8 hours.

Four experiments were performed to examine the effect of activity and fluids on kanamycin absorption and excretion. Five volunteers were selected and a standard dose of 0.5 Gm. of kanamycin sulfate was used in all 4 tests. Blood samples were drawn at 0, 1, 3, 8, and 24 hours; a pretest urine sample was taken and urine collected in time units of from 0 to 3, 3 to 8, 8 to 16, and 16 to 24 hours. These samples were all assayed for kanamycin activity. Aliquots of all samples were frozen and retained until the completion of the 4 experiments when they were assayed together to minimize the effects of assay variability. All urine samples were examined for albumin content and formed elements such as casts and pus cells. In experiment 1, the subjects were kept on absolute bed rest; in experiment 2, the subjects were fully ambulated; in experiment 3, fluid intake was limited to 600 ml. per day for 24 hours prior and during the experiment; and in experiment 4, fluids were forced in excess of 4,000 ml. per day for 24 hours prior and during the experiment.

TABLE I. EFFECT OF SOME ENVIRONMENTAL FACTORS ON KANAMYCIN SERUM LEVELS FOLLOWING INTRAMUSCULAR INJECTION OF 0.5 GM. KANAMYCIN SULFATE

	BED REST	AMBULATION	FLUIDS LIMITED TO 600 ML.	FLUIDS IN EXCESS OF 4,000 ML.
Experiment No.	1	2	3	4
No. subjects	5	5	5	5
		Micrograms/Ml.		
Hours 0	0	0	0	0
1	24.2 (17-31)	22.8 (16-30)	23.4 (17-35)	22.8 (12-33)
3	16.4 (13-20)	20.3 (16-25)	18.6 (16-20)	19.4 (16-22)
8	3.4 (2.2-4.1)	3.5 (3.1-5.4)	4.2 (3.6-5.0)	5.1 (4.4-6.2)
24	0	0	0	0

The kanamycin serum levels obtained in these experiments are presented in Table I. The concentrations were essentially the same and were not influenced by the factors which modified each experiment. The amount of kanamycin excreted in the urine is presented in Table II. As with the serum concentrations, no significant differences in the total amount of kanamycin in the urine were found among the 4 experiments. When fluids were limited to

600 ml. per 24 hours, it would appear that the reduced volume of urine is also associated with a different pattern of kanamycin excretion, even though the total amount excreted was essentially identical with other experiments. The average amount of kanamycin in the urine during the 0- to 3-hour period was substantially lower, and the amount during the 16- to 24-hour period higher, than the amounts found in corresponding samples collected in other tests.

TABLE II. THE EFFECT OF SOME ENVIRONMENTAL FACTORS ON THE URINARY EXCRETION OF KANAMYCIN FOLLOWING THE INTRAMUSCULAR INJECTION OF 0.5 GM. OF KANAMYCIN SULFATE

	BED REST	AMBULATION	FLUIDS LIMITED TO 600 ML.	FLUIDS IN EXCESS OF 4,000 ML.
Experiment No.	1	2	3	4
No. subjects	5	5	5	5
Average 24 hr. urine volume	1,369 ml.	1,191 ml.	800 ml.	3,364 ml.
		Milligrams		
Hours				
0-3	228.4 (160-268.8)	202.3 (85.6-294.6)	154.0 (34.5-212.1)	203.4 (153.6-275.4)
3-8	180.2 (130-245.1)	213.2 (136.4-291)	214.3 (171.6-313.2)	231.7 (179.2-263.5)
8-16	60.2 (25-96)	56.9 (36.7-79.9)	63.3 (47.2-76.3)	64.9 (45.5-81.0)
16-24	8.5 (3-14.9)	13.1 (4.5-22.1)	36.7 (10.7-99.1)	11.5 (8.2-14.9)
Average 24 hr. Kanamycin excreted	477.3	485.5	468.3	511.5

Diuresis with fluids, as evidenced by an average urine excretion of 3,300 ml. in experiment 4, did not materially influence the amount of kanamycin excreted in the time intervals studied. The total amount of kanamycin found in the urine was approximately 90 per cent of the dose injected and was fundamentally identical with the experience reported earlier in this paper. Again as in earlier experiments, 90 per cent of the kanamycin excreted during the first 24 hours occurred during the time period of 0 to 8 hours.

TABLE III. SUMMARY OF URINARY FINDINGS IN FIVE SUBJECTS RECEIVING 0.5 GM. KANAMYCIN SULFATE INTRAMUSCULARLY WEEKLY FOR 4 WEEKS

TIME PERIOD	NO. SAMPLES	W.B.C. hdf			HYALINE CASTS	URATES	PHOSPHATES	Ca OXALATE
		0-2	2-4	4 OR MORE				
0 hr.	20	6	1	3	0	0	2	0
0-3 hr.	20	6	2	3	3	1	2	0
3-8 hr.	20	8	5	3	5	0	3	0
8-16 hr.	20	9	4	1	4	2	3	1
16-24 hr.	20	8	4	2	1	0	1	2

In the experiments described above, 25 urine samples were collected during each test period. The microscopic examination of the centrifuged sediment from these samples revealed an apparent quantitative change in observed hyaline casts (Table III). No casts were found in 20 pretest urines; hyaline casts were found in subsequent urine samples in the following order: 0 to 3

hours, 3/20; 3 to 8 hours, 5/20; 8 to 16 hours, 4/20; 16 to 24 hours, 1/20. The greater number of casts were observed in samples obtained during or shortly after the highest antibiotic concentration.

Side Reactions.—Eighty-nine patients or volunteers have received 1 to 10 doses of kanamycin by intramuscular injection for periods of 1 to 5 days. The side reactions observed are summarized in Table IV. All patients reported slight stinging or an aching pain at the site of injection which lasted for a few minutes to as long as 5 or 6 hours. This discomfort was very mild in most subjects and comparable to that experienced after the intramuscular injection of penicillin. This local reaction was never sufficient to interfere with continued therapy.

TABLE IV. REACTIONS TO KANAMYCIN THERAPY

KANAMYCIN DOSAGE	NO. OF PATIENTS	ADMINISTERED	LOCAL REACTION	PAIN REACTION AND NO. OF PATIENTS	URINARY FINDINGS	OTHER
0.25 Gm. (1 dose)	10	I. M.	0	Slight at time of injection in 10	0	Ambulatory
0.50 Gm. (1 dose)	30	I. M.	Redness in 1	Slight at time of injection in 30	*	Ambulatory
1.00 Gm. (1 dose)	10	I. M.	Redness in 1	Slight at time of injection in 10; muscle soreness in 2; local tenderness in 3	0	Ambulatory
0.50 Gm. every 12 hr. (6 doses)	15	I. M.	Redness in 2; induration in 1	Slight at time of injections in 10; muscle soreness and lameness in 1	1 to 3 hyaline casts/hdf in 5 patients	Ambulatory
0.50 Gm. every 6 hr. (4 doses)	5	I. M.	0	Slight at time of injection in 5	0	Ambulatory
0.50 Gm. every 12 hr. (8 doses)	1	I. M.	0	Slight at time of injection in 1	0	Hospitalized
0.50 Gm. every 12 hr. (10 doses)	12	I. M.	Redness in 2; induration in 1	Slight at time of injection in 12	1 to 2 hyaline casts/hdf in 1 patient	Hospitalized
0.50 Gm. every 6 hr. (10 doses)	1	Oral	0	0	0	Hospitalized

*For details see Table III.

Some urinary observations have already been presented. Of those not previously discussed, 5 of 15 normal volunteers had hyaline casts in the urine following the administration of 3.0 Gm. of kanamycin sulfate over a period of 3 days. The casts disappeared from the urine 48 hours after the discontinuation of kanamycin. One hospitalized patient with a large abscess excreted casts after 5 Gm. of kanamycin over a 5-day period; these disappeared 72 hours after kanamycin therapy was discontinued. In all instances where hyaline casts were observed in the urine, there was no evidence of elevated blood nonprotein nitrogen or albuminuria.

The chemical composition or the significance of the hyaline casts seen in the urine and associated with parenteral kanamycin therapy is unknown. It appears that these casts may be correlated positively with the concentration of kanamycin in the urine. More frequent examinations of urine following kanamycin administration give a higher incidence of casts, particularly if these examinations are performed on samples collected in the first 8 to 16 hours.

SUMMARY AND CONCLUSIONS

1. Kanamycin, a new antibiotic derived from *Streptomyces kanamyceticus*, is readily absorbed from parenteral sites. The peak concentration of kanamycin occurs at approximately one hour after administration, following which there is a rapid decline of serum concentration over the subsequent 12 hours.

2. The average minimum serum concentration of antibiotic following the administration of 0.5 Gm. of kanamycin every 6 hours was 10 μg/ml.; every 8 hours, 5 μg/ml.; every 12 hours, 0.9 μg/ml. The average high serum concentration of kanamycin was approximately 30 μg/ml. regardless of the time interval between injections.

3. Approximately 90 per cent of a given dose of kanamycin can be recovered in urine during the first 24 hours after injection with most of the total being excreted in the first 8 hours.

4. The absorption and 24-hour excretion of kanamycin could not be influenced by bed rest, ambulation, fluids limited to 600 ml. per 24 hours or forced to 4,000 ml. per 24 hours.

5. Hyaline casts were more frequently observed in urine samples collected in the first 16 hours after the injection of kanamycin. All patients experienced mild discomfort at the injection site which did not interfere with further medication.

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EFFECT OF TOTAL BODY X-IRRADIATION ON THE ANTIBODY RESPONSE OF MONKEYS

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MOST of the studies concerned with the effect of ionizing radiations on antibody formation have been conducted in rabbits, rats, and mice.[1, 2] To our knowledge there have been no published data on the effect of irradiation on the immune response of primates in general or monkeys in particular. It is the purpose of this report to describe the effect of total body x-irradiation on the antibody response of monkeys.

MATERIALS AND METHODS

A total of 38 young adult monkeys (1½ to 3 years old, *Macaca mulatta*) were used in these studies. These were divided into 3 groups. Group A (22 monkeys) was inoculated subcutaneously at 3 weekly intervals with 0.5 ml. of commercial triple-typhoid vaccine. In addition, these animals received intravenous injections of a 20 per cent suspension of washed sterile sheep erythrocytes by 2 different immunization schedules during the same typhoid-immunizing period. Nine monkeys were given 3 weekly injections of 0.5, 1.0, and 1.0 ml., respectively (Schedule 1). Thirteen monkeys were given 5 injections of 0.5, 1.0, 1.0, 1.0, and 1.0 ml., respectively (Schedule 2), at 3- or 4-day intervals. All Group A monkeys then received 450 r total body x-irradiation by a previously described method[3] 10 or 12 days *after* the last antigen injection. In brief, a 250 kv. Quadrocondex therapy machine was used at 15 Ma. with a filter of 0.5 mm. copper and 1.0 mm. aluminum. Each animal was irradiated separately while being rotated at 1.3 r.p.m. 1 meter from the x-ray tube. The monkey was in an upright position within a cylinder 20 inches long having an inside diameter of 6 inches. Masonite plates were fitted into each end of the cylinder to prevent undue movement of the monkey during exposure. The exposure rate was 23 r per minute. The wall of the cylinder was of polyethylene, 5/16 inch thick, containing carbon black and was perforated by holes ½ inch in diameter. These were uniformly distributed as 2 holes per square inch of surface area. The density of the polyethylene was 0.93, but with 40 per cent of the wall replaced by holes, the effective density of the cylinder was 0.54. A Victoreen r meter was used before and after each irradiation to measure the dosage being delivered. The meter was one of a paired set calibrated by the Bureau of Standards for use in this project. The dosage in air outside and inside the cylinder was the same within the limits of error imposed by the technique of measurement. This lack of differential may be due to absorption by the wall of the cylinder equaling scatter in the air inside the cylinder. No other object that might be a source of scatter was within 1 meter of the tube.

Group B consisted of 9 monkeys inoculated with typhoid vaccine as above. Four monkeys received sheep erythrocytes by Schedule 1, and 5 by Schedule 2. All Group B animals were irradiated as above with 450 r 24 hours *before* the first antigen injection.

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Group C consisted of 7 control monkeys immunized as above (Schedule 1, 4 animals; Schedule 2, 3 animals) but not irradiated. All monkeys were bled prior to immunization, and at weekly intervals thereafter up to 12 weeks. Typhoid H and O tube agglutinations were carried out by standard methods.[4] The overnight refrigeration technique of Graham and associates[5] was used in the hemolysin titrations.

Eight Group A monkeys and 3 Group C monkeys (controls) were given booster injections of 0.5 ml. typhoid vaccine, subcutaneously, and 1.0 ml. 20 per cent sheep erythrocytes, intravenously, 6 months after irradiation. Four Group B monkeys were given the same booster injections 4 months after irradiation. The antibody response of these animals was followed as described above.

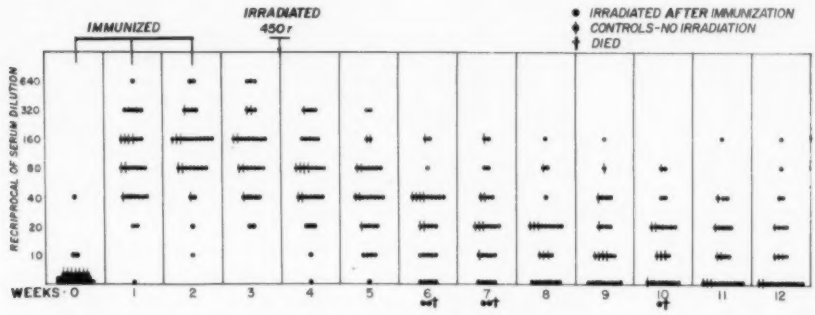

Fig. 1.—Effect of total body irradiation 10 to 12 days after immunization of typhoid H agglutinations in monkeys.

RESULTS

In Group A, which was irradiated *after* immunization, no significant differences from control animals were noted in the H and O typhoid or hemolysin titers, nor was there any difference in hemolysin titers in animals immunized by Schedules 1 and 2. The antibody curves with all 3 tests were similar to those depicting the H agglutinin titers in Fig. 1. All of the monkeys in this group exhibited their peak titers by the time they were irradiated; irradiation did not alter the rate or degree of antibody decline as compared to controls.* Five of the 22 monkeys died as a result of irradiation, but showed no significant antibody differences from their irradiated or control counterparts at the same time intervals. In contrast, monkeys in Group B irradiated 24 hours prior to their first immunizing injection, did show effects of the insult. As can be seen in Table I, none of the 9 irradiated monkeys showed any H agglutinins 1 week after the first immunizing injection, whereas all of the 7 control animals exhibited titers from 1:40 to 1:160 at this time. By the second postirradiation week, and just prior to the third immunizing injection, 4 (Nos. 1, 3, 5, and 6) of the 9 test animals still showed no rise in H agglutinins although the remaining 5 exhibited titers approximating those observed in the controls. During the third week one of the monkeys (No. 3) which had previously shown no titer died, and of the remaining 8 monkeys

*Statistical analysis was carried out using the "t" test for the significance of the difference between two means.

TABLE I. EFFECT OF TOTAL BODY X-IRRADIATION (450 R) GIVEN 24

GROUP	MONKEY NO.	H AGGLUTINATION TITER WEEKS AFTER FIRST INJECTION OF ANTIGEN 0	1	2	3	4	5	6	7	8
Irradiated before immunization (Group B)	1	0	0	0	0	0	0	10	0	0
	2	0	0	40	80	80	80	20	20	20
	3	0	0	0	D					
	4	0	0	160	160	160	80	80	40	40
	5	0	0	0	20	20	20	20	10	10
	6	0	0	0	80	80	D			
	7	0	0	40	160	320	160	80	80	80
	8	0	0	20	20	20	10	10	10	20
	9	0	0	80	160	320	80	80	80	40
	Totals		0/9†	5/9	7/8	7/8	6/7	7/7	6/7	6/7
Controls (not irradiated)	10	0	80	160	80	80	20	20	20	10
	11	0	160	80	40	40	40	40	10	10
	12	0	80	160	160	80	160	40	40	20
	13	0	160	80	160	40	40	20	20	20
	14	0	40	40	80	80	80	40	40	20
	15	0	160	320	320	80	80	40	20	10
	16	0	160	160	320	320	160	160	160	80
	Totals		7/7	7/7	7/7	7/7	7/7	7/7	7/7	7/7

D = died.
ND = not done.
*T = thousand.
†Numerator = number showing titer; denominator = total number.

only 1 (No. 1) failed to show any antibodies 21 days after irradiation. In fact, except for a transient 1:10 titer 6 weeks postirradiation, this animal showed no antibody response during the 8-week observation period. All other monkeys exhibited H agglutinin titers similar to the controls between the third and eighth postirradiation week. One other monkey (No. 6) which had shown a 1:80 titer at 4 weeks died during the fifth week and was lost to the study.

A similar delay in O agglutinin formation as a result of irradiation was observed in the monkeys in Group B (Table I). None of the 9 irradiated monkeys showed any O agglutinin titer at the end of the first week, while titers from 1:40 to 1:160 were observed in all 7 control animals. The subsequent appearance of O agglutinins in the irradiated monkeys followed a pattern very similar to that established by H agglutination. By the third postirradiation week all of the monkeys showed O agglutinins except No. 1 which had also failed to produce H agglutinins at this interval as noted above. A transient O titer of 1:10 was observed in the sera of this monkey at 4, 5, and 6 weeks postirradiation.

The hemolysin response of the Group B monkeys was also delayed following irradiation, and with but minor exceptions, the results were similar to those obtained with typhoid agglutinations. For example, in Monkey 1 (Table I) H and O agglutinins appeared late, were transient in nature, and were present in only very low titer (1:10). In this same monkey, a transient but significant increase in hemolysin titer was observed between the first and second weeks only. However, all other monkeys in Group B demonstrated delayed H and O agglutinin and hemolysin titers to about the same degree.

HOURS BEFORE IMMUNIZATION ON ANTIBODY RESPONSE OF MONKEYS

O AGGLUTINATION TITER WEEKS AFTER FIRST INJECTION OF ANTIGEN									HEMOLYSIN TITER WEEKS AFTER FIRST INJECTION OF ANTIGEN								
0	1	2	3	4	5	6	7	8	0	1	2	3	4	5	6	7	8
0	0	0	0	10	10	10	0	0	1T	4T*	4T	2T	1T	100	100	100	ND
0	0	40	80	80	80	40	20	20	50	50	100	32T	32T	32T	16T	8T	ND
0	0	0	D						0	0	50	D					
0	0	80	160	320	160	80	40	40	100	100	2T	8T	8T	8T	16T	8T	ND
0	0	0	10	20	20	20	20	20	50	50	500	32T	64T	64T	32T	16T	ND
0	0	0	40	80	D				0	50	50	50	4T	D			
0	0	40	160	160	160	80	160	80	50	50	50	1T	2T	32T	64T	64T	16T
0	0	20	20	20	20	20	20	10	2T	1T	2T	8T	256T	512T	128T	512T	64T
0	0	160	320	320	160	80	80	80	50	50	2T	16T	32T	32T	16T	8T	16T
	0/9	5/9	7/8	8/8	7/7	7/7	6/7	6/7		1/9	3/9	7/8	7/8	6/7	6/7	6/7	
0	160	160	40	80	40	20	20	20	100	4T	64T	64T	64T	16T	16T	8T	8T
0	160	160	80	40	40	40	20	10	100	64T	256T	256T	128T	64T	32T	64T	64T
0	80	160	160	160	80	20	40	20	100	16T	64T	128T	32T	16T	8T	4T	8T
0	80	80	80	80	40	40	20	20	0	64T	64T	2T	500	1T	100	50	50
0	40	40	80	80	80	80	40	40	0	1T	2T	500	100	50	100	50	50
0	160	320	160	80	80	40	20	10	50	4T	2T	2T	1T	1T	100	500	500
0	160	160	160	160	160	80	80	80	0	4T	4T	2T	1T	500	2T	500	100
	7/7	7/7	7/7	7/7	7/7	7/7	7/7	7/7		7/7	7/7	7/7	7/7	7/7	7/7	7/7	7/7

Booster injections of typhoid and sheep red cell antigens 4 or 6 months after irradiation resulted in similar antibody rises in both irradiated and control animals. The pattern of the response was the same when measured by H and O agglutination and hemolysin titrations. It is apparent that sufficient regeneration of the antibody forming apparatus had occurred in these monkeys to allow normal secondary antibody responses.

DISCUSSION

In an atomic era one of the many questions that arise would be in relation to any potential modification of the immune response following total body x-irradiation. It is obvious that controlled studies with human beings would not be feasible or morally just. It could be assumed, however, that controlled studies with primates such as monkeys could offer results which probably closely parallel what might be anticipated in the human.

These studies have indicated that total body x-irradiation with 450 r, which is below the $LD_{50/30}$ of about 550 r,[3] can modify the antibody response of monkeys under certain conditions. When the irradiation preceded by 24 hours immunization with typhoid antigen and sheep erythrocytes, there was a definite delay in antibody formation for the first 2 to 3 weeks. Irradiation 10 to 12 days after completion of immunization did not appreciably alter the antibody curves. Monkeys given booster injections 4 or 6 months after irradiation responded in a manner similar to controls. These results are in agreement with previous publications,[1, 2] concerned with other species, that antibody formation is most markedly suppressed when irradiation is given shortly before immunization.

Seven of 31 irradiated monkeys died during the course of the study. All exhibited symptoms and hematologic changes characteristic of radiation sickness.

The antibody response in the 7 monkeys was variable; 3 showed good titers prior to death, whereas titers in the remainder were considered to be fair to poor.

CONCLUSIONS

Total body x-irradiation with 450 r resulted in a temporary suppression of the antibody response of monkeys when administered 24 hours prior to beginning of immunization. No such effect was noted following x-irradiation in previously immunized monkeys. This would suggest that an already immunized individual would fare better, immunologically, following heavy total body irradiation than one immunized subsequent to exposure.

We are indebted to Joann Sparks, Marilyn Fisher, and Egon Nossal for technical assistance.

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BROMSULPHALEIN MOVEMENT IN DECOMPENSATED PORTAL CIRRHOSIS

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IN 1925 Rosenthal and White[1] introduced the disulfonate derivative of phenoltetrabromphthalein (Bromsulphalein or BSP) into clinical use as a liver function test, and with the modification of Mateer and associates[2] it is generally accepted as the most sensitive indicator of hepatic damage in the nonjaundiced patient.

A considerable amount is known regarding the distribution and rate of removal of BSP from the blood stream. When injected intravenously in the normal subject, the dye is removed from the blood at a constant disappearance rate until very low values are reached (below 0.2 mg. per cent).[3] After a short delay BSP appears in high concentration in the bile and continues to be excreted for some time after blood concentrations are negligible. Variable amounts are excreted by the kidneys, and concentrations of the dye can be detected in the gastrointestinal tract, fat, and muscle; further, using S^{35}-labeled BSP, Brauer and associates[4] estimated that 80 per cent of the total dye removed from the circulation in dogs could be accounted for on the basis of hepatic extraction.

However, in patients with decompensated portal cirrhosis it is not known whether there is a rapid distribution of BSP into the ascitic compartment. In such cases a considerable portion of total body weight may be represented by ascitic fluid—ranging upward to 30 per cent in severe cases. The dosage of dye administered in the performance of the BSP test is computed on the basis of weight (5 mg. per kilogram) on the theory that this reflects circulating fluid volume, and it would appear important to know the rate of movement of BSP into and out of the ascitic compartment. It is possible that weight fluctuation such as produced by a recent paracentesis could alter the test without corresponding changes in hepatic function. In order to ascertain this effect, measurements were made of BSP movement in patients with decompensated portal cirrhosis.

MATERIALS AND METHODS

Four patients with a typical history and physical findings of decompensated portal cirrhosis were used in these studies. All had a history of alcoholism, findings of spider angiomata, marked to moderate ascites, and markedly elevated Bromsulphalein retention. Two patients had had bouts of hepatic coma, one surviving, and the other coming to autopsy. The remaining two patients had suffered severe hematemesis from esophageal varicosities but had recovered.

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Bromsulphalein concentrations were measured by a modification of the method of Gaebler.[5] Serum and ascitic fluid were used as blanks and in the preparation of the standard curves.

For easy sampling of the ascites, polyethylene catheters were introduced into the peritoneal space. Serial samples of blood and ascitic fluid were obtained following intraperitoneal and intravenous injection of BSP.

To investigate the passage of BSP from the blood into ascitic fluid, 600 mg. of BSP was introduced intravenously in two patients by slow drip over a period of 2½ hours. The blood levels of BSP were kept between 30 and 60 per cent during this time, and ascitic fluid and blood specimens were taken serially for 5 hours.

The movement of BSP out of the ascitic compartment was measured in 3 cases after the intraperitoneal injection of 150 mg. of the dye. Blood specimens were taken at frequent intervals as were samples of peritoneal fluid which were removed at a site distant from that of injection. Long-range sampling of the ascitic fluid (by needling of the peritoneal space) was continued for 5 days to a week in the patients studied.

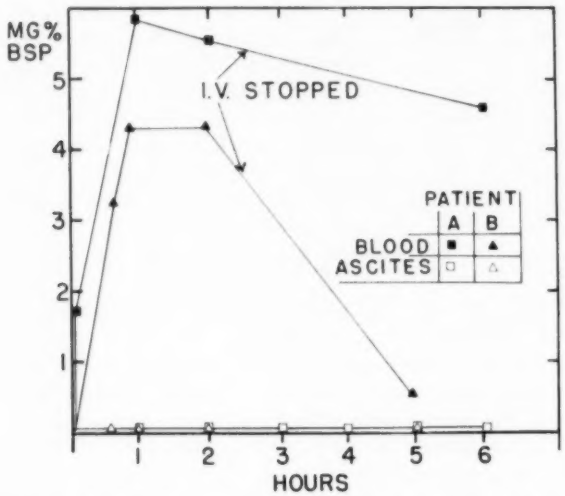

Fig. 1.—Continuous intravenous administration of 600 mg. of BSP with repeated sampling of blood and ascites.

RESULTS

Fig. 1 graphically demonstrates the result of the slow infusion of 600 mg. of BSP intravenously over a period of 2½ hours. It is to be noted that during the interval of infusion the blood concentration remained between 30 and 60 per cent, and after 5 hours significant blood levels were present in both cases. Despite this, BSP was not found in demonstrable amounts in the peritoneal fluid at any time within the sampling period.

Fig. 2 shows disappearance rates of BSP injected intraperitoneally in 4 patients. It is to be noted that the curves are remarkably flat. Repeated blood sampling during this time did not reveal demonstrable BSP to be present in the blood.

In Fig. 3 is shown the ascitic fluid concentrations following intraperitoneal injection. These values are given for a period of from 4 to 6 days in contrast to the short-term observations shown in Fig. 2. In one patient a significant concentration (7 per cent) was encountered after 6 days, while in the other the level had fallen to 3 per cent within 4 days.

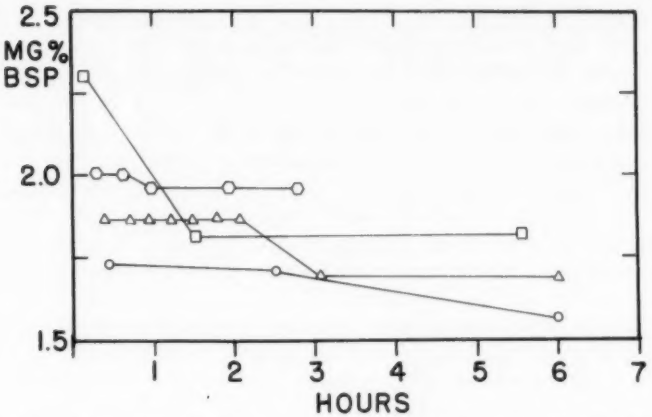

Fig. 2.—Short-term sampling of ascitic fluid following intraperitoneal administration of 150 mg. of BSP.

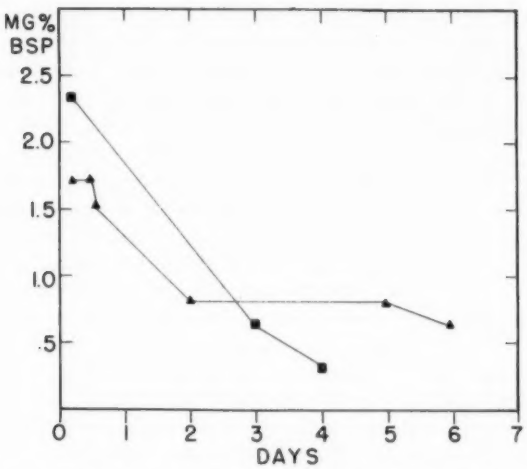

Fig. 3.—Long-term sampling of ascitic fluid following intraperitoneal administration of 150 mg. of BSP.

DISCUSSION

Our results would suggest that BSP transfer from the blood to the ascitic compartment or vice versa is at such a slow rate that the weight of the ascites

should be considered in the calculation of the dosage of the dye. Failure to allow for this weight would result in the administration of excess dye with the subsequent elevation of blood levels.

BSP injected into the ascitic fluid, or intravenously, binds to the serum albumin fraction, the proteins of these two compartments being qualitatively similar. This binding has been reported by various workers[4, 6] and we have observed the BSP to consistently migrate with the albumin fraction of either serum or ascitic fluid during electrophoretic separation. The movement of the BSP in or out of the peritoneal space probably then mirrors the migration of the albumin fraction.

There is abundant evidence that ascitic fluid is in dynamic equilibrium with the circulating blood. Studies with tritium-labeled water[7] indicate that the water content of ascitic fluid enters and leaves the peritoneal space at a rapid rate, approximately 40 to 60 per cent every hour. The turnover of other blood substances such as protein occurs at a somewhat slower rate. Schoenberger and associates[8] using tagged albumin report exchange rates between the plasma and ascites of between 0.4 and 0.65 Gm. per hour. McKee[9] investigated the turnover of serum albumin in the "ascitic dog" prepared by constriction of the superior vena cava and reported complete transferral in from 2 to 7 days. Thus, it is probable that in the decompensated cirrhotic, the turnover of albumin is most variable but occurs in from 1 to 7 days.

The rate with which BSP leaves or enters the peritoneal space depends on the factors influencing the migration of albumin. This transfer is so slow that for the purposes of the clinical measurement of BSP retention, the peritoneal space is "closed." Fig. 2 demonstrates this and it can be seen that within a 2-hour period the disappearance curve of BSP is flat. This observation has clinical application in the measurement of ascitic fluid volume. Our technique consisted of the intraperitoneal injection of 150 mg. of BSP followed by measurement of dye concentration in the ascites after a period of ½ hour (to allow for mixing). The sampling is done at a site removed from injection. It is interesting that in our autopsy case the ascitic volume performed two days prior to death agreed quite well with the measured volume (16,000 ± 300 ml.). This "closed" ascitic fluid compartment should also be considered in the calculation of the amount of dye to be injected in the performance of the standard BSP liver function test. Gross estimation of weight will prove satisfactory for clinical purposes. However, for a more accurate assay an ascitic fluid volume can be determined by measuring the dilution of Bromsulphalein or other dyes such as phenolsulfonphthalein or Evans blue. The weight of the ascites can then be calculated from the specific gravity and the absolute "dry weight" of the patient determined. Changes in the BSP retention using this technique will more faithfully measure changes in the function of the polygonal cells of the liver.

CONCLUSIONS

1. The movement of Bromsulphalein in and out of the peritoneal space is quite slow in cases of decompensated portal cirrhosis. This is probably governed

by its linkage to the serum albumin and the multiplicity of factors which govern the migration of protein.

2. It is suggested that in the performance of the Bromsulphalein liver function test an allowance be made for the estimated weight due to ascites.

3. Bromsulphalein is a satisfactory substance with which to measure ascitic fluid volume.

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CEREBROSPINAL FLUID TRANSAMINASE ACTIVITY IN ACUTE AND CHRONIC NEUROLOGIC DISEASES

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THE clinical usefulness of serum glutamic oxalacetic transaminase determinations in cardiac, hepatic, and pulmonary diseases is now well established. Brain tissue contains a relatively large amount of transaminase activity; therefore, elevations of activity in the cerebrospinal fluid would logically be expected following cerebral infarction and other acute destructive nervous system lesions; yet, there has been considerable controversy regarding the clinical usefulness of this test in neurologic diseases.[2, 5, 7]

A simplified colorimetric method for assay of glutamic oxalacetic transaminase was described by Reitman and Frankel[6] for use as a routine laboratory procedure. Utilizing this method an attempt was made to evaluate further the possible diagnostic and prognostic significance of the enzyme activity in both serum and cerebrospinal fluid (CSF). Serial determinations on patients with cerebral infarctions and correlation of the autopsy findings with the CSF and serum transaminase activity were attempted in order to determine the possible correlation with the size of the area of destruction, as well as the optimal time to obtain the CSF transaminase estimation for maximal clinical usefulness.

MATERIALS AND METHODS

Selection of Patients.—This report is based upon glutamic oxalacetic transaminase determinations in 35 control subjects and 126 patients with various neurologic diseases: cerebrovascular accidents (39), seropositive syphilis (25), inactive neurosyphilis (14), multiple sclerosis (16), convulsive disorders (10), toxic encephalopathy (4), neuropathy associated with diabetes mellitus (3), and miscellaneous neurologic diseases (15). Seven of the patients with cerebrovascular accidents had postmortem examinations.

Methods.—Spinal fluid and serum transaminase activities were determined by a modification of the Reitman-Frankel[6] method. The technique is based on the production of oxalacetate from α-ketoglutarate and subsequent conversion to a 2,4-dinitrophenylhydrazine which is measured colorimetrically in an alkaline medium.

All specimens were refrigerated from the time they were obtained until the assays were performed. In most cases serum was separated from the whole blood within 10 hours after refrigeration and the determination was accomplished within 72 hours. Cerebrospinal fluid specimens were analyzed only if they were free of blood.

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Two-tenths milliliter of spinal fluid or serum was mixed with 1.0 ml. of α-ketoglutarate (2 mM. per liter) and DL-aspartate (200 mM. per liter) both of which had previously been incubated in a water bath at a constant temperature (37° C.) for 10 minutes. After 60 minutes' incubation, 1 ml. of 2,4-dinitrophenylhydrazine (1 mM. per liter) was added to stop the reaction. This mixture was alkalinized with 0.4 N sodium hydroxide after 20 minutes at room temperature. Thirty minutes later the optical density of the solution was measured at 505 mμ using water as a blank in a Beckman spectrophotometer, Model DV. Transaminase activity of spinal fluid in Reitman-Frankel units was obtained from a standard curve prepared from pyruvate, 2 mM. per liter. Significance of the differences between the control and various patient groups was evaluated by rank sum test.[9]

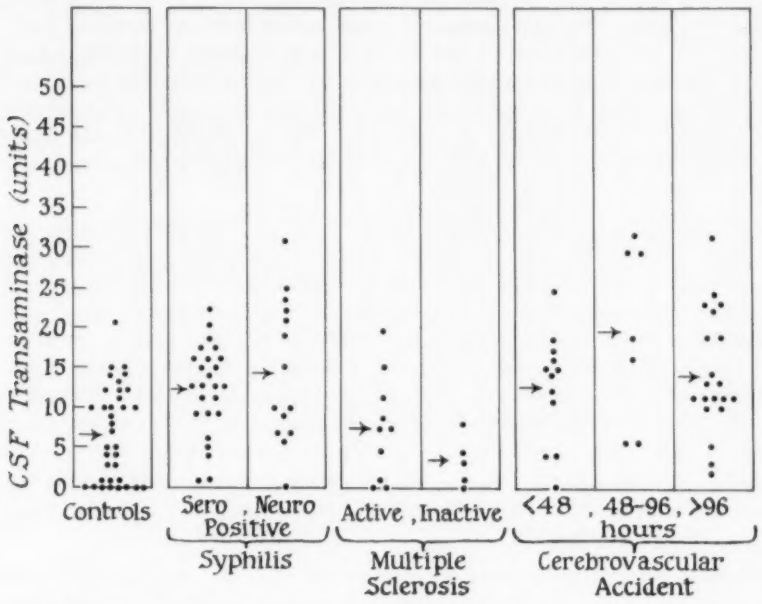

Fig. 1.—Cerebrospinal fluid transaminase values in the control group, inactive syphilis, multiple sclerosis, and cerebrosvascular accidents (nonhemorrhagic). The arrows represent mean values.

RESULTS

Normal Values.—Transaminase determinations were done on the cerebrospinal fluid of 35 patients without apparent neurologic or systemic disease who had lumbar punctures for spinal anesthesia incident to minor surgical procedures, or who had psychiatric conditions in which organic disease was eventually excluded. Simultaneous serum transaminase levels were measured in 28 of these patients. Values ranging from 0 to 21 units were found in the CSF with a mean of 6.8 units (see Fig. 1). The standard deviation of 5.8 units indicates that 95 per cent of all the control values would be included between 0 and 17.4 units. CSF transaminase activity above 17.4 units was considered to be abnormal. The serum transaminase ranged from 0 to 36 units with a mean of 15.5 units.

Cerebrovascular Accidents.—These patients were divided into 3 groups on the basis of the length of time between the onset of symptoms and the time when the cerebrospinal fluid was obtained for analysis. Twelve patients in whom spinal fluid was obtained within 48 hours after onset of their illness showed CSF transaminase values ranging from 0 to 25 units with a mean of 12.5 units. The serum transaminase in these patients ranged from 0 to 51 units with a mean of 16.3 units. Seven patients in whom spinal fluid was obtained between 48 and 96 hours after the onset of the illness revealed CSF transaminase values ranging from 0 to 32 units with a mean of 19.7 units. The serum transaminase in these patients ranged from 0 to 87 units with a mean of 25.6 units. Twenty patients in whom spinal fluid was obtained longer than 96 hours after the onset of the illness showed CSF transaminase ranging from 2 to 31 units with a mean of 14.0 units. The serum transaminase in these patients ranged from 0 to 101 units with a mean of 18.5 units. The distribution of CSF values is presented in Fig. 1. Clinical findings, cerebrospinal fluid transaminase activity, and autopsy results in 7 cases are summarized in Table I.

Syphilis.—This group included blood seropositive-treated patients and those with neurosyphilis without elevation of CSF protein or cell count which were, therefore, considered to be inactive. CSF transaminase activity in 25 specimens from patients with treated syphilis having had positive blood serology showed a mean of 12.2 units in the CSF. In this group 24 patients had serum transaminase levels with a mean of 13.3 units. Fourteen patients with inactive neurosyphilis showed a mean of 14.2 units in the CSF. Twelve of these patients had serum transaminase values ranging from 5 to 48 units with a mean of 15.5 units. (See Fig. 1.) There was no significant difference in the serum transaminase levels in patients with treated blood seropositive syphilis and those with inactive neurosyphilis, as compared to normal individuals in the control group.

Multiple Sclerosis.—Patients with multiple sclerosis were divided into two categories: those with active disease who had developed clinical signs of progression within one month of the determination (10 cases) and 5 patients in whom the disease was considered to be inactive. The CSF in the active group showed a mean of 7.5 units, and the mean in the inactive group was 3.4 units. There was no significant difference between the CSF values in the active and inactive cases or the control group. Serum transaminase values in this active group showed a mean of 26.1 units and the inactive group a mean of 12.2 units. A fulminating case of acute disseminated encephalomyelitis with a left hemiplegia, internuclear ophthalmoplegia, and marked mental deterioration showed elevated CSF transaminase activity of 74 units 10 days after the onset but was not included in the statistical analysis because of the marked difference from other members of this group.

Seizures.—Ten patients with recurrent motor seizures of from 1 to 17 years' duration without evidence of active disease of the brain were studied.

CSF transaminase levels were determined within 48 hours after the last attack in 5 cases and 4 to 36 days after the last seizure in the other 5 patients. The range was from 0 to 15 units in both groups with a mean of 5.7 units, showing no significant difference from the control group. Five serum determinations were also within normal limits.

Toxic Encephalopathy.—Two patients in uremia with convulsion were found to have CSF transaminase levels of 10 and 12 units. A patient with

TABLE I. SUMMARY OF CLINICAL, AUTOPSY, AND CSF TRANSAMINASE FINDINGS IN ACUTE CEREBRAL INFARCTION*

PATIENT AND AGE	CLINICAL FINDINGS—DATED FROM ONSET PRIOR TO DEATH	POSTMORTEM FINDINGS	CSF TRANSAMINASE VALUES—REITMAN-FRANKEL UNITS
1. L. K. (62 yr.)	Sudden aphasia with partial remission, 14 days. Sudden right hemiparesis with right Babinski sign, 5 days	Large ischemic infarct in the distribution of the left middle cerebral artery with evidence of processes of two distinct ages correlating with the clinical history. There was hemorrhage into the wall of a markedly sclerotic left middle cerebral artery	Day 3: 32 Day 4: 20 Day 7: 10 Day 8: 14 Day 9: 50 Day 12: 50 Day 14: 33
2. F. S. (68 yr.)	Abrupt and persistent loss of consciousness with left hemiplegia and deviation of eyes to left, left pupil larger than right, 10 days	Moderate sized ischemic infarction secondary to thrombosis of right middle cerebral artery	Day 5: 2 Day 9: 10
3. J. B. (77 yr.)	Sudden right hemiplegia and aphasia, 11 days	Large ischemic infarction in distribution of left middle cerebral artery. Marked cerebral atherosclerosis	Day 1: 15 Day 3: 10 Day 5: 7 Day 7: 32 Day 9: 20
4. I. D. (45 yr.)	Sudden left hemiplegia and unconsciousness, 2 days. Known rheumatic heart disease, 20 yr., and atrial fibrillation, 9 yr.	Large ischemic infarction in distribution of right anterior and middle cerebral arteries secondary to thromboembolus in right internal carotid artery. Mural thrombi in left auricular appendage	Day 1: 30
5. F. McD. (65 yr.)	Abrupt and persistent coma with bilateral Babinski's sign, 6 days. History of remote left hemiparesis, 1 yr.	Large recent ischemic infarction in distribution of partially recanalized right middle cerebral artery	Day 1: 17
6. G. M. (32 yr.)	Sudden left hemiparesis and left lower facial weakness. Known periarteritis nodosa with cardiac involvement, 1 day	Small ischemic infarct, right middle cerebral distribution. Multiple visceral infarcts including heart	Day 1: 0 (Serum transaminase on same day—17 units)
7. L. S. (61 yr.)	Sudden aphasia, right hemiparesis, and somnolence, 9 days	Moderate sized hemorrhagic infarction in distribution of left middle cerebral artery secondary to thrombosis of markedly atherosclerotic left internal carotid artery	Day 1: 25 Day 3: 11 Day 8: 7

*The size of the infarct is defined as follows: small infarctions indicate an estimated less than 100 Gm. of tissue necrosis, moderate sized infarcts refer to 100 to 200 Gm. of tissue necrosis, and large infarcts refer to more than 200 Gm. of tissue necrosis.

delirium tremens and no clinically evident liver disease was found to have a CSF transaminase level of 17 units. An abnormally high CSF transaminase of 29 units was found in one patient the day after admission with acute diabetic acidosis.

Neuropathy Associated With Diabetes Mellitus.—CSF transaminase values in 3 such cases showed values of 9, 12, and 15 units which are considered to be within normal range.

Miscellaneous.—Fifteen patients with neurologic diseases were included in this group, representing the following disorders: Guillain-Barré syndrome, optic neuritis, amyotrophic lateral sclerosis, cervical disc, myasthenia gravis, botulism, parkinsonism, and cerebellar degeneration. In all of these, the CSF transaminase activity was below 15 units and considered to be not elevated. Two out of three brain tumors (gliomas) showed elevations of 24 and 36 units. One 47-year-old patient with pneumoencephalographic evidence of cerebral atrophy had a CSF transaminase level of 17 units.

DISCUSSION

Opinion varies as to the clinical value of the cerebrospinal fluid transaminase activity in cases of cerebral infarcation as well as other destructive neurologic lesions. There have been reports of significant elevations of CSF transaminase in experimentally produced cerebral infarctions in dogs[8] as well as in patients with cerebrovascular disease, head injury, convulsive disorders, or degenerative disease of the central nervous system.[1] Others have reported a lack of correlation between the presumed pathologic process, the severity of the disease, and CSF transaminase level.[4]

Examination of the results obtained in this study and correlation with autopsy findings indicate that large cerebral infarctions which terminate fatally usually produce significant elevations in CSF transaminase activity rising during the first 10 days of the illness. Small infarcts are unlikely to produce significant spinal fluid increases in the enzyme content. These findings do not constitute proof of the hypothesis that brain tissue necrosis is the major factor in producing elevated CSF transaminase, but the results are consistent with this concept. There was no quantitative relationship between serum and CSF transaminase levels. The CSF transaminase activity did not correlate with CSF protein content or the proximity of the lesions to the subarachnoid space or ventricles.

Individuals having multiple sclerosis with recent clinical evidence of activity of the disease showed no significant CSF transaminase difference from the normal group nor did the CSF transaminase values in the active group show any significant difference from those who were designated as clinically inactive.

It is important to stress that wide variations in the results from any of the groups studied makes it impossible to draw significant conclusions in an individual case regarding the extent of damage, etiology, or activity of the

disease process. Refined statistical analysis showed several significant differences, however, the relatively small number of patients in the "homogeneous" groups did not warrant such interpretation. In cases where the diagnosis is clinically apparent, an elevated CSF transaminase during the course of the disease substantiates the clinical impression. As larger numbers of cases are tested, it is becoming more apparent that the CSF transaminase level is not of clinical importance in evaluating diseases of the nervous system.

SUMMARY AND CONCLUSIONS

1. Estimation of the transaminase activity of the cerebrospinal fluid was done in 35 control subjects and 126 patients with neurologic disease by a simplified colorimetric method described by Reitman and Frankel.

2. The autopsy findings in 7 patients with ischemic cerebral infarctions of thrombotic and embolic etiology are correlated with the transaminase determinations indicating significant elevations in transaminase activity, sometime during the first 10 days following a large cerebral infarction. Infarctions involving less than an estimated 200 Gm. of cerebral tissue at autopsy showed poor correlation with the transaminase values.

3. Analysis of the cerebral spinal fluid transaminase values in groups of patients with treated blood seropositive syphilis, inactive neurosyphilis, convulsive disorders, multiple sclerosis, toxic encephalopathy, and other neurologic disorders resulted in no evidence of clinical usefulness of such determinations in patients with a variety of diseases of the nervous system.

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MYELOTOXICITY, NEPHROTOXICITY, AND ERYTHROPOIETIC STIMULATING FACTOR PRODUCTION IN ANIMALS TREATED WITH THE AMINONUCLEOSIDE OF PUROMYCIN

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THE aminonucleoside, 6-dimethylaminopurine-3-amino-d-ribose, has been found to induce an experimental nephrosis in rats and, by selective renal perfusion experiments, unilateral kidney disease in dogs.[1-3] In addition, this compound has been investigated as a possible cancer chemotherapeutic agent.[4] The ability of the aminonucleoside to produce an experimental nephrosis in rats was confirmed in this laboratory. However, the drug also was found to have a myelotoxic action, as shown in rats and rabbits by experiments in which iron utilization, reticulocytes, bone marrow morphology, and in vitro hemin synthesis were studied. Likewise, assays for erythropoietic-stimulating factor(s) were carried out, since the role of the kidney in the regulation of erythropoiesis is still controversial.[5-7] It is the purpose of this paper to present the results of these experiments.

METHODS

Male Sprague-Dawley rats were used for all experiments and were fed a Purina rat chow diet with water ad libitum. In most experiments the rats were given 14 daily subcutaneous injections (0.3 ml. per 100 grams of body weight) of a 0.5 per cent solution of 6-dimethylaminopurine-3-amino-d-ribose.* Fiegelson and his associates[2] had previously reported the production of an experimental nephrosis by this amount of aminonucleoside. Controls were given injections of saline. An anemia was produced in certain groups of animals by 4 daily subcutaneous injections of 0.6 ml. of a 0.625 per cent phenylhydrazine solution. In later studies the entire dose of aminonucleoside was given in two injections 24 hours apart. Twenty-four hours after the last injection the animals were sacrificed and special studies were carried out as described in the following sections.

Routine hematologic counts were made by standard methods. Reticulocyte counts were obtained by counting 1,000 red blood cells after staining with methylene blue. Bone marrow preparations were stained with Wright's stain and the number of nucleated erythroid cells per 1,000 nucleated cells was noted.

The iron utilization of the peripheral red cells was measured 18 hours following intravenous injection of 1 μc of Fe^{59} as ferrous citrate diluted in saline.† One milliliter of heparinized blood obtained by cardiac puncture was used for measurement of radioactivity.

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†The Fe^{59} had a mean specific activity of 2.5 mc. per milligram.

The percentage of utilization was calculated on the basis of a blood volume of 5 per cent body weight.[8] All radioactivity counts were made in a well scintillation counter.* The percentage of radioiron present in the liver, spleen, and bone marrow, when measured, was performed as described by Rambach, Alt, and Cooper.[9]

Assay for erythropoietic-stimulating factor(s) was carried out by the method of Jacobson and associates utilizing 125 to 150 Gm. Sprague-Dawley rats starved for 96 hours.[10] Water was not limited. At approximately 30 and 54 hours after the institution of starvation 2 ml. of test plasma or saline was injected into the tail vein of each of 6 animals comprising the assay group. Eighteen hours prior to sacrifice 1 μc of Fe^{59} citrate was given intravenously, and the iron utilization was determined as described in the previous paragraph.

In 3 experiments the in vitro synthesis of hemin following the addition of aminonucleoside was measured by a modification of the Thomas method utilizing Fe^{59}. Details of the hemin synthesis method will be reported in another publication.[11] Aminonucleoside was added to normal plasma in amounts calculated to be comparable to that given to rats in a single day of the 14- and 2-day experiments.

Statistical analysis of the results, when performed, was made by Student's "t" distribution test with a determination of the p value.

RESULTS

1. *General.*—The aminonucleoside-induced nephrosis in rats described by Fiegelson and co-workers[2] was produced in these experiments. The animals developed marked ascites, an elevated blood urea nitrogen (BUN) and proteinuria. The mean values for BUN determinations in 4 groups of animals receiving equal test injections were as follows: saline, 14.1 mg. per cent; aminonucleoside, 150.0 mg. per cent; aminonucleoside plus phenylhydrazine, 162.2 mg. per cent; phenylhydrazine alone, 14.3 mg. per cent. The mean weight gain for the animals receiving saline was 89 Gm. The mean gain for animals receiving aminonucleoside and aminonucleoside plus phenylhydrazine was 64 and 40 Gm., respectively. At autopsy the kidneys were found to be pale and swollen, and microscopic examination revealed the presence of a nephrosis with tubular damage, the presence of casts, and connective tissue infiltration by lymphocytes.

Preliminary observations revealed a depression of the reticulocyte counts of the animals given the aminonucleoside; consequently, more detailed studies of bone marrow function were performed.

2. *Myelotoxic Action.*—The peripheral blood counts are presented in Table I. These results showed the decrease in reticulocyte counts in the animals which received aminonucleoside (Table I and Fig. 1). The mean value of these reticulocyte counts was 0.9 per cent compared to the 3.7 per cent found in control animals given saline. Statistically, this decrease was significant with a p value < 0.001. It should be noted that the animals which were given phenylhydrazine in addition to the aminonucleoside had a mean reticulocyte count of 7.9 per cent as compared to a value of 48.0 per cent in animals that received phenylhydrazine alone. Statistically, this was also very significant with a p value of < 0.001. A more severe anemia was produced by the same amount of phenylhydrazine in the aminonucleoside-treated animals. The mean hematocrit was 20 per cent in aminonucleoside-treated animals compared to 31.7 per cent

*Nuclear Research & Development Well Counter, Model CS-600.

TABLE I. PERIPHERAL BLOOD AND BONE MARROW VALUES IN EXPERIMENTAL ANIMALS

DETERMINATIONS	SALINE	14-DAY AMINONUCLEOSIDE	PHENYLHYDRAZINE	AMINONUCLEOSIDE + PHENYLHYDRAZINE
Reticulocyte (per cent)	3.7 ± 0.4 (34)*	0.9 ± 0.2 (22)	48.0 ± 3.3 (23)	7.9 ± 1.7 (20)
Red cell count (millions per c.mm.)	7.31 ± 0.31 (24)	7.44 ± 0.05 (12)	-	3.62 ± 0.07 (11)
Hemoglobin (Gm. per 100 ml. blood)	13.7 ± 0.3 (24)	13.1 ± 0.2 (12)	-	5.6 ± 0.3 (11)
Hematocrit (volume of packed red cells in ml. per 100 ml.)	41.8 ± 0.2 (24)	40.0 ± 1.0 (12)	31.7 ± 1.0 (23)	20.0 ± 1.0 (11)
Mean corpuscular volume (cubic microns)	58.9 ± 2.0 (24)	54.4 ± 1.5 (12)	-	46.0 ± 1.6 (11)
Mean corpuscular hemoglobin (micromicrograms)	19.2 ± 0.6 (24)	17.7 ± 0.5 (12)	-	15.6 ± 0.3 (11)
Mean corpuscular hemoglobin concentration (per cent)	32.7 ± 0.3 (24)	32.7 ± 0.6 (12)	-	27.9 ± 0.5 (11)
Platelets (thousands per c.mm.)	701.5 ± 42.2 (24)	1208.4 ± 107.7 (12)	-	1175.9 ± 123.9 (11)
Leukocyte count (thousands per c.mm.)	15.0 ± 1.0 (24)	13.4 ± 1.4 (22)	-	25.4 ± 3.9 (11)
Neutrophils (per cent)	20.0 ± 1.5 (24)	27.0 ± 3.4 (12)	-	39.0 ± 3.1 (11)
Stab (per cent)	1.2 ± 0.2 (24)	0.5 ± 0.1 (12)	-	2.0 ± 0.7 (11)
Eosinophil (per cent)	0.9 ± 0.2 (24)	1.0 ± 0.3 (12)	-	1.0 ± 0.2 (11)
Basophil (per cent)	0.1 ± 0.04 (24)	0 (12)	-	0 (11)
Lymphocyte (per cent)	75.0 ± 1.6 (24)	65.0 ± 3.5 (12)	-	54.0 ± 2.5 (11)
Monocyte (per cent)	2.8 ± 0.3 (24)	7.0 ± 0.7 (12)	-	4.0 ± 0.7 (11)
Normoblast (per cent)	0 (24)	0 (12)	-	3.5 ± 1.3 (11)
Erythroid elements in marrow (per cent)	15.0 ± 1.1 (16)	3.2 ± 0.5 (26)	37.2 ± 4.8 (14)	7.6 ± 0.6 (21)

*Values are reported as the mean and standard deviations of the mean. The numbers in parentheses refer to the number of animals used for each observation.

in the animals treated with phenylhydrazine alone. The mean corpuscular hemoglobin decreased in animals treated with aminonucleoside. In the animals given phenylhydrazine plus aminonucleoside there was a decrease in the mean corpuscular hemoglobin concentration to 27.9 per cent compared to 32.7 per cent in animals given saline or aminonucleoside alone. The mean corpuscular volume also decreased.

The platelet counts increased in the animals receiving aminonucleoside. A leukocytosis was produced in the group of animals that received phenylhydrazine in addition to the aminonucleoside. There were minor differences in the differential counts in the different groups, but these changes were not thought to be significant.

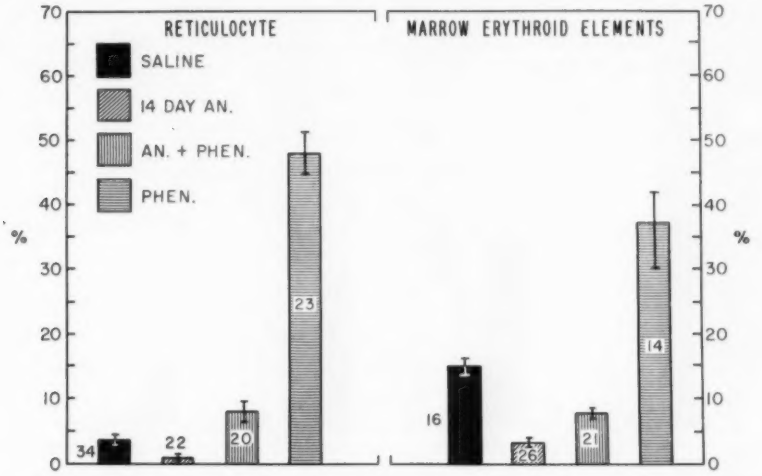

Fig. 1.—Percentage of reticulocytes and marrow erythroid elements under different experimental conditions. AN refers to aminonucleoside and phen to phenylhydrazine injections. The number of experimental animals used for each observation is given in the appropriate bar. The vertical lines in each bar outline the standard deviation of the mean.

The results of these experiments pointed to possible marrow damage; therefore, in the next experiment the percentage of nucleated erythroid cells in bone marrows was calculated (Table I and Fig. 1). The erythroid activity of animals given aminonucleoside was decreased when compared to control animals. Statistically, this decrease was significant with a p value < 0.001. As shown in Table I the bone marow of the aminonucleoside animals was unable to respond normally to the stimulus of the anemia produced by phenylhydrazine. When values for the marrow erythroid activity of these animals receiving aminonucleoside and phenylhydrazine were compared statistically to values for animals receiving phenylhydrazine alone, the p value was < 0.001.

Further evidence for erythroid depression was found in the histologic examination of the spleen of the experimental animals and in a comparison of the

ratio of the spleen weight to the body weight of the animals. Microscopic examination revealed markedly diminished erythroid activity in the spleens of animals given aminonucleoside when compared to the spleens of control animals. When the ratio of the weight of the spleen to body weight was calculated the following values were obtained: saline controls 0.006; aminonucleoside 0.003; aminonucleoside plus phenylhydrazine 0.002; phenylhydrazine alone 0.014. Thus, the spleen to body weight ratios for the aminonucleoside animals were one-third and one-half that of the control animals and one-fifth to less than one-sixth of the value for the animals which received phenylhydrazine alone.

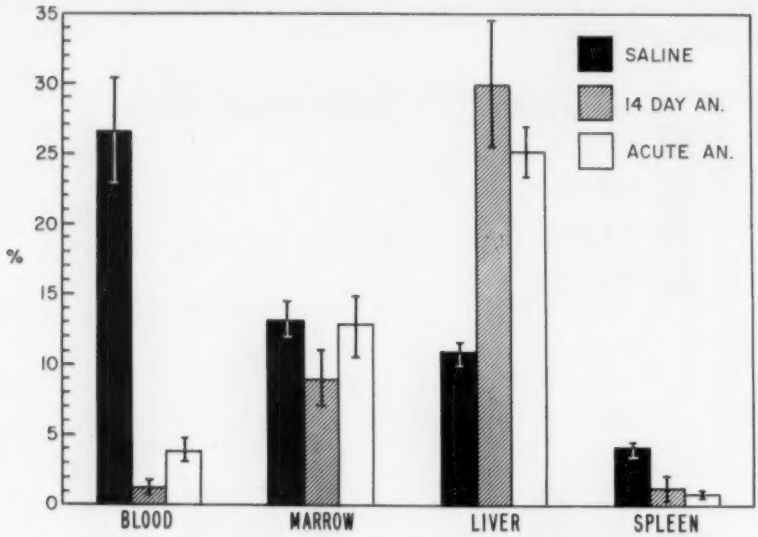

Fig. 2.—Percentage of 18-hour Fe^{59} incorporation in blood, bone marrow, liver, and spleen of experimental animals. AN refers to aminonucleoside-treated animals. The vertical line in each bar outlines the standard deviation of the mean. Six animals were used in the saline and 14-day aminonucleoside groups. Eleven animals were used in the acute aminonucleoside group.

More direct evidence for bone marrow damage was found in the measurement of 18-hour erythrocyte iron utilization. The results of these experiments are graphically portrayed in Fig. 2. In addition to the utilization of iron by the peripheral erythrocytes, the percentage of radioiron present in the bone marrow, liver, and spleen 18 hours after intravenous injection is noted. In animals treated with aminonucleoside alone a mean utilization of 1.20 per cent of the injected radioiron was present in the peripheral erythrocytes in comparison to 26.56 per cent in fed control rats. In the animals that received phenylhydrazine in addition to the aminonucleoside only 0.83 per cent of the radioiron was utilized in the peripheral blood. Less radioiron was present in the bone marrow and spleen of the animals receiving aminonucleoside whereas a higher percentage of the iron appeared in the liver.

Since some of the changes of bone marrow function could have been due to depression of erythropoiesis by the retention of waste products, studies were made in animals receiving the entire dose of aminonucleoside over a period of 24 hours. Under these circumstances the BUN was within normal limits and there was no histologic evidence of renal disease. However, only 1.1 per cent of the nucleated cells in the bone marrow belonged to the erythroid series. When compared to saline controls this is very significant with a p value of < 0.001. The results of radioactive iron utilization are shown in Fig. 2. It is seen that the 18-hour iron utilization by the peripheral red cells was markedly depressed to 3.9 per cent. Less iron was also present in the spleen while the percentage appearing in the liver was increased when compared to animals receiving saline.

Further evidence for the direct marrow toxicity by aminonucleoside is found in 3 experiments of in vitro hemin synthesis. The results are recorded in Table II. In 2 of the 3 experiments, 5 mg. of added aminonucleoside significantly depressed hemin synthesis. Twenty milligrams of aminonucleoside depressed hemin synthesis in each experiment.

TABLE II. IN VITRO HEMIN SYNTHESIS WITH ADDED AMINONUCLEOSIDE

EXPERIMENT	NORMAL SERUM (COUNTS PER 5 MIN.)*	5 MG. AN/3 ML. (COUNTS PER 5 MIN.)*	20 MG. AN/3 ML. (COUNTS PER 5 MIN.)*
1	127.6	113.8 (88.0)	24.5 (19.0)
2	1014.0	260.4 (25.0)	59.9 (5.8)
3	2520.0	872.0 (34.0)	193.0 (7.6)

AN = Aminonucleoside.

*Counts per 5 minutes per milligram of hemin synthesized. Numbers in parentheses refer to the percentage of hemin synthesized with added aminonucleoside using the value of normal serum in each experiment as 100 per cent.

TABLE III. ASSAY FOR ERYTHROPOIETIC-STIMULATING FACTOR(S) UNDER VARIOUS EXPERIMENTAL CONDITIONS

ASSAY MATERIAL	ASSAY ANIMAL	NUMBER OF ANIMALS	PER CENT Fe^{59} UPTAKE*
Saline	Starved rat	87	4.4 ± 0.2
Phenylhydrazine rat plasma	Starved rat	16	13.3 ± 1.7
Phenylhydrazine + aminonucleoside rat plasma	Starved rat	32	7.3 ± 0.5

*Values are reported as the mean and standard deviation of the mean.

3. *Erythropoietic-Stimulating Factor Production.*—The erythropoietic-stimulating factor content of the plasma of two groups of animals was measured indirectly by the erythrocyte iron utilization of the starved rat. These values were compared to control animals that had received saline injections. The results are recorded in Table III. The plasma of animals pretreated with aminonucleoside and then made anemic with phenylhydrazine caused a Fe^{59} utilization of 7.3 per cent in starved assay animals, whereas the plasma of animals treated with phenylhydrazine alone increased the iron utilization of starved rats to 13.3 per cent. The 7.3 per cent uptake compared to the 13.3

per cent was statistically significant with a p value of < 0.001. The 7.3 per cent uptake is also a statistically significant increase when compared to the 4.4 per cent iron utilization of assay animals receiving saline injections.

DISCUSSION

The parent compound of the aminonucleoside used in these experiments is the antibiotic Puromycin. This antibiotic has been used as a trypanosomide, and its aminonucleoside as a possible cancer chemotherapeutic agent.[4, 12] The aminonucleoside of Puromycin has been found to have nephrotoxic properties and has been used experimentally to produce acute nephrosis, chronic glomerulonephritis, and, by selective perfusions, unilateral kidney disease.[1-3, 13] The nephrotoxicity has been confirmed in this laboratory. In addition, Selye has reported the production of interstitial pancreatitis and osteitis fibrosa-like changes in the bones of rats receiving this compound.[14] He felt on the basis of histopathologic examination that the bone marrow elements were replaced by a fibrous tissue that contained many osteoblasts.

The present studies definitely show a myelotoxic action of the aminonucleoside. In animals receiving aminonucleoside there was a depression of the erythroid elements of the bone marrow and a reticulocytopenia. There was a marked inhibition of radioiron utilization by these animals. They showed a decreased ability to respond to the stress of an anemia produced by phenylhydrazine. The studies also showed that the myelotoxic effect could be produced in acute experiments before the onset of azotemia. This myelotoxic action must be considered if the aminonucleoside is used as a cancer chemotherapeutic agent.

The pathogenesis of myelotoxic action is unknown. Recant and her associates[13] have studied the mechanism of the nephrotoxic action of the aminonucleoside and suggest that there is a causal relationship between adenosinetriphosphate (ATP) formation and the ability of the aminonucleoside to induce experimental renal disease. Experiments have not been carried out to determine whether the myelotoxic action is due to ATP inhibition. It is of interest, however, that hemin synthesis by in vitro marrow suspensions, as shown in this study, can be inhibited by the aminonucleoside. It is probable, therefore, that the aminonucleoside has the ability to inhibit several enzyme systems.

The markedly decreased mean corpuscular hemoglobin concentration in animals receiving both aminonucleoside and phenylhydrazine is of interest. These studies showed that hemin synthesis in vitro was impaired. With the stimulation of phenylhydrazine, more cells were placed in the circulation and, since hemin synthesis was probably impaired in vivo, a decrease in the mean corpuscular hemoglobin concentration would result. Whether this is the entire explanation is not known.

The role of the kidney in control of erythropoiesis and the hypothesis of marrow utilization of erythropoietic-stimulating factor(s) are at present subject to differences of opinion. Jacobson and associates[5] found that following

nephrectomy animals were no longer able to respond to the administration of cobalt by the production of these factor(s).[5] This view has been disputed by Erslev[6] and Mirand and Prentice[7] who demonstrated "erythropoietin" production in their nephrectomized animals using different stimuli. Erslev, however, did find decreased activity of these substances 72 hours after nephrectomy when azotemia was marked. In regard to utilization of erythropoietic-stimulating factor(s) it has been postulated that increased levels will be found in situations where the marrow is unable to respond. Thus, increased levels have been found in patients with refractory anemia and by Stohlman in animals following marrow ablation by x-rays.[15] However, further evidence is needed before this hypothesis can be accepted, since other investigators have shown increased levels in polycythemia and hemolytic anemias where markedly hyperplastic marrows are present.[16-18]

The present studies showed that with the advanced renal disease produced by aminonucleoside the animals were less able to produce erythropoietic-stimulating factor(s) when injected with phenylhydrazine. The plasma from these animals produced less Fe^{59} utilization, when assayed in the starved rat, than did the plasma obtained from nonuremic rats with phenylhydrazine-induced anemia. Further, if the hypothesis of marrow utilization of erythropoietic-stimulating factor(s) is correct, one would have expected higher levels in the animals that received aminonucleoside plus phenylhydrazine, since these animals had a marked decrease in bone marrow erythroid activity and a more severe anemia than did the animals given phenylhydrazine alone. The results of these experiments would provide some evidence in favor of the hypothesis of Jacobson and associates[5] that the kidney plays a role in the production or activation of erythropoietic-stimulating factor(s). However, certain reservations must be made since the plasma of the animals receiving aminonucleoside plus phenylhydrazine did cause an increase in the Fe^{59} utilization of assay animals when compared to control assay animals receiving injections of saline. Further experiments are planned in an attempt to elucidate the mechanism of the myelotoxicity of aminonucleoside and the relationship of bone marrow activity to erythropoietic-stimulating factor(s) production.

SUMMARY

1. The aminonucleoside of Puromycin was used to produce a toxic nephrosis in rats.

2. The material was found to be myelotoxic. Erythropoiesis was decreased as demonstrated by reticulocytopenias, decreased marrow and spleen erythropoiesis, and decreased radioiron red cell utilization. In vitro hemin synthesis by rabbit bone marrow was inhibited by the aminonucleoside.

3. Animals with nephrosis showed decreased ability to produce erythropoietic-stimulating factor(s).

4. The relationship of the kidney and of bone marow cellularity in levels of erythropoietic-stimulating factor(s) is briefly discussed.

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THE HEMODYNAMIC EFFECTS OF ISOPROTERENOL IN MAN

With Observations on the Role of the Central Blood Volume

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ALTHOUGH isoproterenol (Isuprel*) is a widely used drug both clinically and as an experimental tool, there is limited information available regarding its hemodynamic effects in normal man.

The present study was undertaken to evaluate the evidence that the so-called central blood volume acts as a permissive factor limiting cardiac output responsiveness.[1-3] It had previously been demonstrated that although atropine-induced tachycardia produced a striking increase in cardiac output in the recumbent human subject, it failed to do so when the central blood volume was depleted in the upright position.[3] In view of these observations it appeared of interest to study, under similar conditions of a depleted central blood volume, the effect of an agent normally having a positive inotropic action. From this has developed a more general study of the hemodynamic effects of isoproterenol on the normal human subject.

MATERIALS AND METHOD

The subjects were normal male university students, 18 to 27 years old. The cardiac output was determined by the dye dilution method, as modified from Doyle and his colleagues,[4, 5] but employing superior vena caval injections. In all studies a relatively steady state, as determined by constancy of arterial pressure and pulse rate on monitored records, was achieved before the time of the cardiac output determinations. Stroke volume was calculated from pulse rate during the interval of the cardiac output determinations. Arterial and central venous pressures were obtained by strain gauge and recorded on a photographic multichannel recording system. The fourth intercostal space 5 cm. below the angle of the sternum was the reference point for all pressure determinations in both postures. Mean circulation time and "central blood volume" were calculated by the Hamilton method from dye dilution curves.[4, 5] Statistical analysis was performed according to the methods described by Snedecor.[6]

RESULTS

Two groups of 6 subjects each were studied, one in the recumbent posture and the other in the passive 60 degree head-up tilt posture. In all studies control cardiac output determinations in the appropriate posture were made. Ten

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*Trade name, Winthrop Laboratories, Inc., New York, N. Y.

to fifteen minutes following the control observation an intravenous infusion of isoproterenol (Isuprel) (1 μg contained in a 2 ml. physiologic saline solution per minute) was begun and a second cardiac output determined 5 to 10 minutes after beginning the infusion and 3 to 5 minutes after a steady state had been achieved. In the studies performed with subjects at 60 degree head-up tilt, the control and isoproterenol observations were made during the same interval following assumption of the tilted posture.

TABLE I. HEMODYNAMIC EFFECTS OF ISOPROTERENOL IN RECUMBENCY AND HEAD-UP TILT

SUBJECT		CARDIAC OUTPUT (L./MIN./M.²)	PULSE RATE (BEATS/MIN.)	STROKE VOLUME (ML.)	MEAN ARTERIAL PRESSURE (MM. HG)	CENTRAL VENOUS PRESSURE (CM. H_2O)	MEAN CIRCULATION TIME (SECONDS)	"CENTRAL BLOOD VOLUME" (ML.)
Recumbency								
J. K.	C	3.2	82	76	72	2.0	15.4	1,599
	I	4.1	86	94	72	−1.5	10.8	1,462
M. M.	C	3.2	66	97	82	4.5	17.2	1,820
	I	4.4	88	100	72	3.0	11.4	1,678
H. H.	C	2.4	58	75	85	4.0	21.1	1,530
	I	3.7	74	90	81	2.0	13.5	1,496
P. W.	C	2.5	46	90	72	3.5	22.6	1,563
	I	3.9	60	108	73	2.0	16.2	1,750
R. R.	C	2.7	60	101	83	1.5	18.1	1,827
	I	4.2	67	140	81	0.5	14.0	2,182
J. M.	C	3.5	80	86	79	1.5	14.1	1,607
	I	5.9	92	124	78	0.5	9.9	1,878
Average		2.9	65	88	79	2.8	18.1	1,658
		4.4	78	109	76	1.1	12.6	1,741
p		<0.01	<0.01	<0.02	>0.05	<0.01	<0.01	>0.4
60 Degree Head-Up Tilt								
A. M.	C	2.6	82	58	74	0	15.9	1,261
	I	2.6	100	48	70	−0.5	13.0	1,034
P. O.	C	2.8	108	51	76	3.0	11.1	1,014
	I	3.1	112	51	85	3.0	10.6	1,085
A. Q.	C	2.7	86	56	67	2.0	15.3	1,224
	I	3.0	96	57	67	1.5	13.3	1,215
C. H.	C	2.1	76	54	82	1.0	19.2	1,301
	I	2.9	84	65	76	1.0	16.9	1,535
B. F.	C	2.2	86	49	81	5.0	19.0	1,340
	I	3.3	104	62	81	5.0	14.4	1,555
B. L.	C	2.1	84	54	72	4.0	18.7	1,410
	I	3.8	102	78	73	4.0	12.9	1,720
Average		2.4	87	54	75	2.5	16.5	1,236
		3.1	100	60	75	2.4	13.5	1,357
p		<0.05	<0.02	>0.05	>0.9	>0.8	<0.02	>0.8

C = control observation; I = during isoproterenol infusion.

The data are summarized in Table I. In each of the recumbent subjects the infusion of isoproterenol resulted in moderate increases in cardiac index, associated in 5 of the subjects with slight increases in pulse rate and moderate elevation of stroke volume. The increase in mean cardiac index and stroke

volume were significant. Central venous pressure fell consistently during isoproterenol infusion. In tilted subjects the increases in cardiac index and stroke volume during isoproterenol infusion were of lesser magnitude, despite similar degrees of elevation in heart rate. Whereas 5 of the 6 individuals studied in recumbency demonstrated an increase in cardiac index of 1.2 L./min./M.^{2}, or greater, in only 1 of the 6 individuals studied during 60 degree tilt did the cardiac output rise by this amount. The differences in the magnitude of cardiac output and stroke volume responses between the two groups were significant ($p < 0.05$ and < 0.01, respectively, for cardiac index and stroke volume). Central venous pressure readings tended to remain unchanged during isoproterenol infusion in the tilted subjects.

Mean circulation time diminished in all of the individuals during isoproterenol infusion, with greater falls occurring in recumbent subjects. Although the changes in central blood volume for the group were not significant, moderate elevations were noted in those individuals demonstrating the largest cardiac output and stroke volume responses. Mean arterial pressure did not change significantly in either group.

DISCUSSION

The cardiac output and stroke volume responses to isoproterenol observed in the recumbent human subject are similar to the hemodynamic alterations produced in animals.[7-12] They also are consistent with previous observations in man utilizing indirect means of estimating cardiac output[13, 14] or dealing with subjects with heart disease.[15-17] The studies reported here demonstrate that in the normal supine subject an increase in cardiac output occurs resulting from the combined effect of augmented stroke volume and elevated heart rate. Of particular interest was the diminished responses in the tilted subject. Arterial pressure remains essentially unchanged in both postures indicating a fall in peripheral resistance.

These data are notable in view of the observations indicating that the central volume of blood plays an important role in limiting or permitting the cardiac output and stroke volume response in the face of suddenly altered circulatory demands.[1-3] In the recumbent posture atrial pressure is relatively high, ventricular filling occurs rapidly and a more than adequate blood volume is available to meet any sudden increase in circulatory demand. In the upright posture the thoracic blood volume is diminished,[18] right atrial pressure falls,[19] and ventricular filling occurs less rapidly.[20] It is under these circumstances that we have observed a diminished response to isoproterenol infusions, even though its action appears to be primarily on the myocardium. The present observations, therefore, offer further experimental support to the thesis that the central venous pool acts as an important factor limiting increases in cardiac output and stroke volume.

Of particular interest is the fact that the limitation imposed on cardiac output in the upright subject was of much greater magnitude when challenged by an agent which primarily increased heart rate (atropine), as compared to one acting on myocardial contractility (isoproterenol). In the tilted subject under the influence of atropine, cardiac output failed to rise and stroke volume

diminished precipitously with the tachycardia, although during similar rate changes in the horizontal subject, stroke volume was effectively maintained during the brief period of observation.[3] During isoproterenol infusion cardiac output increased to some degree, even under the stress of upright posture, and was associated with a slight increase in heart rate while stroke volume remained unchanged or increased in 5 of the subjects. The specific cause for these differences cannot be explained definitively at this time. It would appear possible that the agent with the positive inotropic action (isoproterenol) not only enhances ventricular stroke output, but may concurrently aid ventricular inflow from a depleted central reservoir by direct or indirect action. The work of Wiggers and Opdyke[21, 22] on the greater rate of ventricular relaxation following epinephrine stimulation, as well as recent observations on elastic recoil[23] and active ventricular diastolic suction[24-26] make this mode of action of isoproterenol a plausible one. An alternate explanation for the observed differences is that isoproterenol may enhance venous inflow by direct vasoconstrictor activity simultaneous with its myocardial action. The decrease in central venous pressure observed in our subjects, however, during the isoproterenol infusion, does not lend support to this thesis and would not be expected from pharmacologic studies on this drug.[10]

The fall in central venous pressure associated with the enhanced cardiac output induced by isoproterenol has been observed previously in the heart-lung preparation by Konzett[7] and in human subjects during the initial effect of other cardiac stimulants, such as epinephrine[27] and atropine.[3] Further observations on blood volume shifts and vasomotor activity appear to be required in order to explain this interesting phenomenon.

SUMMARY AND CONCLUSIONS

1. The cardiac output responses to a constant infusion of isoproterenol (Isuprel, isopropyl norepinephrine) have been studied in normal subjects in the recumbent and passive head-up tilt postures.

2. Significant elevations in cardiac output and stroke volume associated with slight increases in pulse rate were observed in recumbent subjects. In tilted (head-up) subjects the minute and stroke volume responses to isoproterenol infusions were of significantly less magnitude, while the pulse rate increases were of similar degree.

3. These data are consistent with the view that as a consequence of the peripheral pooling of blood and depletion of the thoracic reservoirs in the upright posture, there is limitation on cardiac output and stroke volume responsiveness. This effect is less, however, with an agent (isoproterenol) having a positive inotropic action than with one acting predominantly on heart rate (atropine).

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RIBOFLAVIN ACTIVITY IN NORMAL AND LEUKEMIC HUMAN LEUKOCYTES AND NUCLEI

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VARIOUS groups of workers have from time to time investigated the role that riboflavin and its coenzymes, riboflavin monophosphate (FMN) and flavin adenine dinucleotide (FAD), might play in malignant tissues as compared to normal ones. Of particular interest in this regard is the report by Polli[1] that riboflavin may be found in the nuclei of leukemic leukocytes but that it is absent from the nuclei of corresponding normal cells. If correct, then, because of the catalytic functions of flavin coenzymes, one might anticipate gross metabolic differences between the two tissues. It is just this kind of qualitative difference between normal and malignant cells that has been sought by investigators in the cancer field as an aid in designing effective chemotherapeutic agents. In the present report are included our findings with microbiologic measurement of riboflavin in isolated human leukocytes and their nuclei and an analysis of factors which might influence the vitamin activity in such samples.

METHODS

Leukocytes free from contamination by other formed elements have been prepared from peripheral blood by the phytohemagglutinin method of Li and Osgood[2] as modified in our laboratory.[3] White cell yields have been increased by one additional modification of the procedure. By adding a few milliliters of phosphate buffer (1.42 ml. 85 per cent H_3PO_4, 2.0 Gm. sodium acetate, 0.003 Gm. ascorbic acid; make to 1 liter, adjusting with NaOH to pH 7.45)[3] to the sedimented erythrocytes (R.B.C.) and tapping the side of the tube gently, white cells (W.B.C.) which have layered on the red cells shake loose and may be added to those of the supernatant plasma.

Leukocyte nuclei have been prepared by the sucrose-citric acid method of Frazer and Davidson.[4] It is important that each suspension be stained and examined microscopically, for, while intact cells are seldom seen, in some preparations significant quantities of cytoplasm are left adhering to the nuclei. The presence of cytoplasm gives an apparent increase in nuclear riboflavin content, and in this study determinations have been included only when at least 70 per cent of the nuclei were clean and free of cytoplasmic contamination. Total and differential counts on intact cells and on nuclei have been made by procedures already described.[3]

Riboflavin has been determined microbiologically with *Lactobacillus casei* by the acid extraction procedure described by us for whole blood.[5] To ensure that sufficient riboflavin is present for determination, about 50 ml. of blood is drawn from normal donors to determine white cell content and approximately 200 ml. to determine the quantity in W.B.C.

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nuclei. With the elevated counts to be found in leukemic patients, correspondingly smaller blood samples are utilized. Determinations performed in duplicate and triplicate on samples of different size agree within the limits of experimental accuracy. No riboflavin activity was detectable in the phosphate buffer wash of the W.B.C. suspension. Since *L. casei* responds equally well to riboflavin, riboflavin monophosphate, and flavin adenine dinucleotide, it should be understood that the expression of riboflavin activity in this report actually reflects the sum of free vitamin and its coenzymes. Diagnosis of the several forms of leukemia has been by repeated blood and bone marrow studies, and in the majority of the acute cases by postmortem examination.

RESULTS

By application of the phytohemagglutinin technique for isolating white cells from peripheral blood, preparations have been made in which leukocytes consistently make up greater than 90 per cent of the cell population. The purity of the W.B.C. obtained from leukemic bloods is 99.5 per cent (mean), and 95.0 per cent for normal bloods. The cells are well preserved morphologically and are freely suspendable for counting purposes. To determine to what extent erythrocyte contamination might contribute to leukocyte riboflavin values, assays were performed on the erythrocytes of 18 normal individuals and 10 patients with hematologic disorders (leukemia, anemia, and polycythemia vera). A mean of 0.029 μg per 10^9 R.B.C. (S. E., 0.002) was found for both groups. Using this value, we have calculated that the erythrocyte riboflavin contribution to that determined for isolated W.B.C. in the case of the grossest contamination is of the order of only one part in 1,000.

TABLE I. RIBOFLAVIN IN NORMAL AND LEUKEMIC HUMAN LEUKOCYTES

DIAGNOSIS	NUMBER OF INDIVIDUALS	MICROGRAMS RIBOFLAVIN/10^9 W.B.C.			P*
		MEAN	S. D.	S. E.	
Normal	14	2.70	0.52	0.14	---
Lymphocytic leukemia	21	0.98	0.25	0.06	<0.01
Granulocytic leukemia	18	2.35	0.68	0.16	0.13
Monocytic leukemia	2	3.84, 4.89	---	---	---

*P = Probability that deviation of mean from that of normal is due to chance.

The results of W.B.C. riboflavin determinations for normal and leukemic individuals are summarized in Table I. The mean riboflavin content of the leukocytes of patients with lymphocytic leukemia is seen to be significantly less than that found for either normal or granulocytic leukemic individuals, the latter two groups having essentially the same mean. As the differential count showed that 92 per cent of the W.B.C. of patients with lymphocytic leukemia were of the lymphocytic series, one may take the white cell riboflavin values for these individuals as values for (leukemic) lymphocytic cells, similarly for granulocytic leukemia and granulocytic cells. Further evidence that the W.B.C. riboflavin levels are characteristic of the cell type rather than necessarily of the leukemic process comes from several atypical cases that we have studied. Two lymphocytic leukemic patients with unusually low lymphocyte counts in their differential had unusually high riboflavin values. In one of these individuals with a marked eosinophilia (57 per cent), a level

typical of granulocytic cells was found (1.79 μg per 10^9 W.B.C.). On the other hand, in a person with granulocytopenia (99 per cent lymphocytes) of unknown etiology, a typically decreased white cell riboflavin concentration was observed (1.12 μg per 10^9 W.B.C.). The leukocytes isolated from normal bloods, although chiefly of the granulocytic series, are comprised, of course, of a mixed population.

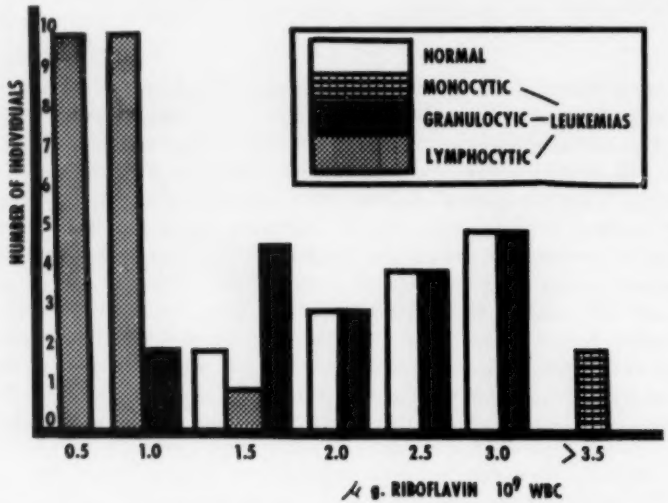

Fig. 1.—Distribution of riboflavin values in human leukocytes.

Reference to the bar graph of Fig. 1 shows that there is an essentially bimodal distribution of riboflavin values for lymphocytes as compared to granulocytes. The two groups overlap, however, in the range of 1.4 to 1.6 μg riboflavin per 10^9 W.B.C. It is interesting that roughly equal numbers of acute and chronic leukemic patients give values in this range and that repeat determinations as much as a year apart agree closely. All the determinations on cells from patients with lymphocytic leukemias are found to cluster at the low end of the graph. Individuals with the granulocytic leukemias give somewhat more variable values and are closely paralleled by the normal group. Only two cases of (acute) monocytic leukemia have been included in this study, but in both instances higher white cell riboflavin values were found than in any other individuals studied.

Because in leukemia one finds that the circulating leukocytes are not only of a single series but also frequently at a particular stage of development, it has been possible to determine whether the riboflavin activity of the cells changes as a function of maturation. By application of regression statistics to a plot of W.B.C. riboflavin versus percentage of blasts in the cell population, it has been found that there is no significant correlation between the maturation state of the leukocyte and its riboflavin content. Similarly,

regression statistics show that there is no significant correlation between W.B.C. riboflavin level and either whole blood riboflavin activity, fasting state, age, or sex of the donor.

Determination of riboflavin in isolated leukocyte nuclei again shows that cells isolated from normal and granulocytic leukemic individuals do not differ from each other (Table II), but leukocyte nuclei from lymphocytic leukemic patients have a decreased riboflavin differing significantly from that for the other two groups at the 5 per cent probability level. It may be seen in the last column of Table II that the lymphocyte nucleus contains approximately twice as much of the cell's riboflavin as does the nucleus of the granulocyte.

TABLE II. RIBOFLAVIN IN NORMAL AND LEUKEMIC HUMAN LEUKOCYTE NUCLEI

DIAGNOSIS	NUMBER OF INDIVIDUALS	MICROGRAMS RIBOFLAVIN/ 10^9 *W.B.C*			P*	PER CENT W.B.C., RIBOFLAVIN IN NUCLEUS
		MEAN	S. D.	S. E.		
Normal	8	0.62	0.22	0.08	---	23.0
Lymphocytic leukemia	10	0.40	0.14	0.04	<0.05	40.8
Granulocytic leukemia	7	0.56	0.12	0.05	0.5	23.8

*P = Probability that deviation of mean from that of normal is due to chance.

DISCUSSION

The data presented here on W.B.C. riboflavin are difficult to compare with those of other investigators because of differences in mode of expression of results, method of determination, and method of isolating the leukocytes and evaluation of their purity. In this study, a close parallel has been found between the riboflavin values in the white cells of normal persons and those with the granulocytic leukemias. This similarity probably reflects the fact that in both groups the leukocyte population is comprised primarily of cells of the granulocytic series, although to a greater degree, of course, in this group of leukemic patients. In contrast, cell populations with high lymphocyte differential counts (as found in patients with the lymphocytic leukemias) give leukocyte riboflavin values which are only one third to one half of those for cells of the granulocytic series. Of particular interest is the observation that this difference in riboflavin content between the granulocytic and lymphocytic series is maintained through the various stages of maturation from the blast forms to the mature cell. Except, therefore, in those cases where the series show some overlap, one may differentiate between the lymphocytic and granulocytic forms of acute leukemia. It may also be possible to distinguish acute monocytic leukemia in this manner, but only two cases have been studied, and any conclusion must be tentative.

We have previously reported the elevation of whole blood riboflavin in patients with the granulocytic leukemias. This observation may be explained by the increased quantity of the vitamin in the granulocyte as compared to that in the lymphocyte. As was the case with whole blood determinations, microbiologic measurement of erythrocyte riboflavin in the present study has given a mean value (31.9 μg per cent) higher than that obtained by fluorometry (22.4 μg per cent).[6, 7]

The findings on nuclear riboflavin activity do not coincide with those of Polli[1] in suggesting any marked difference between the nuclei of normal and leukemic leukocytes. Although the lymphocyte nucleus contains a statistically significantly smaller quantity of riboflavin than does the granulocyte nucleus, it is seen that the major difference in the vitamin content of the two series of cells lies in the cytoplasm. That the granulocyte contains more of the vitamin may reflect, at least in part, its larger volume, the size increase being primarily cytoplasmic.

In light of the studies by Callan,[8] Watson,[9] and others on the porosity of the nuclear membrane, it is possible that some riboflavin has been lost during isolation of the nuclei. If such is the case, then the values reported here may represent only that portion of the nuclear riboflavin which is tightly bound.

SUMMARY

Riboflavin has been determined microbiologically in human leukocytes isolated in high purity by the phytohemagglutinin technique. Determinations on white cells from normal individuals and patients with granulocytic and lymphocytic leukemia have shown the lymphocytic series of cells to contain only one third to one half as much vitamin as do the granulocytic cells. White cell riboflavin correlates neither with age, sex, or fasting state of the donor, nor with whole blood riboflavin level or leukocyte maturity. In view of the latter finding, it is possible in most instances to distinguish between the white cells of the several forms of acute leukemia and thus make a differential diagnosis. Nuclei of lymphocytic cells have a slightly smaller (statistically significant) quantity of riboflavin than granulocytic nuclei, but the major difference between the vitamin content of the two series of cells is in the cytoplasm.

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A STUDY OF EXPERIMENTAL STAPHYLOCOCCAL ENDOCARDITIS IN DOGS

I. Production of the Disease, Its Natural History, and Tissue Bacteriology

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THE study of bacterial endocarditis in experimental animals has been limited over the years by numerous technical difficulties. As a spontaneous infection it has been reported rarely in dogs, sheep, and zoo animals, although it is said to be less rare in the opossum.[1]

The prerequisite for producing experimental endocarditis has usually been deliberate injury to a heart valve, followed by repeated bacterial injections. Occasionally, however, cases have been recorded in rabbits following single injections of large numbers of organisms isolated from human cases.[2] Horses used for the production of antipneumococcal or antimeningococcal serum have occasionally developed bacterial endocarditis.[3] This has occurred mostly after repeated single injections and has been associated with a certain degree of immunity to the organism used. In recent years it has been found that a fairly high percentage of rabbits made hyperimmune to foreign proteins developed endocarditis after repeated injections of bacteria,[4] again suggesting some relationship to the hyperimmune process on the endothelial cells. In 1937, Nedzel[5] used Pitressin as a shock stimulus, following which he injected India ink and bacteria. By this technique he obtained a low incidence of endocarditis. In addition, Nedzel thought he could demonstrate endothelial changes manifested by pseudopods from the endothelial cells and believed this was the area of bacterial localization. He surmised that anoxia and blockage of endothelial cells by India ink were necessary adjuvants to the procedure.

The use of valvular trauma followed by bacterial injections has been the most common method of producing experimental endocarditis. In 1938, Kinsella and Muether[6] passed hooks through the carotid arteries of dogs, cut the aortic or mitral valves, and 30 days later gave injections of 10 ml. of a 24-hour broth culture of streptococci. By this method they were able to obtain a fairly high incidence of bacterial endocarditis. They also noted that large doses of streptococci given orally to 17 such dogs produced endocarditis in 58 per cent. More recently Highman and Altland[7, 8] have discovered that the

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exposure of young rats to altitudes of 25,000 feet on repeated occasions would produce changes suggestive of the nonbacterial endocarditis seen sometimes in human beings. They also noted that if the rats were injected intravenously with bacteria prior to the expected date of these changes pathologically, bacterial endocarditis frequently occurred. They postulated that polycythemia and anoxia may have contributed to the induction of the bacterial endocarditis, although they subsequently discovered that neither factor alone was sufficient. Cortisone, when given prior to the bacterial injections, increased the incidence of bacterial endocarditis.

More recently Lillehei and associates[9, 10] have found that the production of large arteriovenous fistulas in dogs has made them quite susceptible to spontaneous and artificially induced bacterial endocarditis. The required size of the fistula varied with the dogs' age, younger dogs requiring either one iliac plus a femoral fistula, or 2 iliac fistulas, or an aorta-vena cava fistula. In dogs 5 to 10 years old, one iliac or 2 femoral fistulas alone were sufficient to set the stage for endocarditis, either spontaneous or artificial after bacterial injections. One surmises from their work that there is a critical amount of blood flow through the shunt at the time the dog's heart becomes susceptible to endocarditis. The lesions were usually found on the aortic or mitral valves and were occasionally found on the endocardial surface of the arteriovenous fistuli. Of the 10 cases of endocarditis which developed in dogs with large fistulas, 4 were caused by *Aerobactor aerogenes,* 1 by *Streptococcus viridans,* and 1 by *Streptococcus hemolyticus;* cultures were not obtained in the remaining 4 dogs. This is the only method described which has given rise to a high incidence of bacterial endocarditis without direct trauma to the valves themselves.

Roshe and Morrow[11] and Highman and associates[12] have described a method of producing graded aortic insufficiency in dogs weighing 7½ to 15 Kg. with a relatively low operative mortality. Endocarditis followed multiple intravenous bacterial injections. They later showed that 6 dogs developed acute bacterial endocarditis after single injections of either streptococci or staphylococci within 21 days of the operation.[13] The insertion of a Hufnagel valve did not lower the incidence of bacterial endocarditis following injection of bacteria.

METHODS

Production of Aortic Insufficiency.—Healthy male dogs weighing 9½ to 25 kilograms were examined and observed to be normal preoperatively. On the day of surgery the body temperature was reduced to about 32° C. under Nembutal anesthesia. The animal was then placed upon the operating table in the left lateral decubitus position. The temperature usually fell to about 28° C. during the operation. The right side of the chest was shaved, prepared aseptically, and an endotracheal tube inserted. Respirations were maintained by the use of a Jefferson respirator.

The chest was opened in the right fourth intercostal space with an incision extending from the sternum to the tip of the scapula laterally. On most occasions the blood vessels were cauterized although occasionally silk ligatures were used entirely for hemostasis. The internal mammary arteries on the right side were usually sacrificed. Exposure was

very good and access to the superior vena cava and the right side of the heart was obtained with ease. The azygos vein was ligated in most dogs. A heavy silk ligature of 3-0 silk was placed around the superior and inferior vena cavas with the ends passed through plastic tubes to be used as collars. After the injection of several centimeters of 1 to 2 per cent procaine into the pericardial sac, the pericardium was opened. Occasionally the vessels of the pericardium were cauterized; at other times they were ligated with silk. The fat pad about it was dissected free. An umbilical tape was usually placed around the aorta for traction purposes. The heart and aorta were palpated to detect the presence of thrills, but they were never found preoperatively. Fine 1-0 arterial silk sutures were then placed in the root of the aorta approximately one inch above the aortic valve ring. At times a U- or Z-shaped suture line was laid through media and adventitia without tying the ends, but at other times parallel sutures were laid beforehand. The superior and inferior venae cavae were then occluded with the plastic collars and the heart was allowed 30 seconds in which to empty. The aorta was then incised in a linear fashion with a Bard-Parker blade between the previously laid sutures, the incision measuring about 3 mm. A curved steel tube, 3 mm. inner diameter, was inserted through the incision and passed down until one of the aortic cusps was engaged. This gave a sense of resistance with moderate "give." With a rotary motion of the steel tube, its cutting edge removed a disc of valvular tissue 3 mm. in diameter. The instrument was then removed from the aorta and the previously laid sutures were tied. On most occasions hemostasis was good and blood loss was minimal. The superior vena cava was then released slowly and 5-0 arterial silk sutures were used to repair any bleeding defects in the aortic incision. The inferior vena cava was then released. In a few of the dogs the pericardium was left open without sutures but in most two silk sutures were used to approximate the pericardial surfaces loosely. The chest was then closed in layers and the right chest aspirated to remove residual air. In all but one dog (57-931) a diastolic murmur, a diastolic thrill, or both were detected before the animal was removed from the operating table. The dog was then placed by a heater to raise its body temperature. It was given 300,000 units of penicillin subcutaneously for 5 to 7 days and when there had been a lapse in sterile operative technique an additional 0.5 to 1.0 Gm. of streptomycin was given daily for 5 days. The sutures were removed between 7 and 14 days after surgery.

The period of inflow occlusion to the heart rarely exceeded 3 to 4 minutes. Occasionally it was felt necessary to reocclude inflow and reopen the aorta to repeat the procedure because the original procedure failed to give a thrill or the murmur was incorrectly interpreted as being systolic. This resulted in several dogs having discs of tissue removed from 2 cusps. Early in the course of experimentation we attempted the procedure with direct visualization of the valve during cardiac arrest, but a uniform difficulty in restarting the heart caused us to abandon this method.

Operative Complications.—The most common complication of surgery was ventricular fibrillation. Four of 33 dogs died on the operating table. Three deaths were due to spontaneous ventricular fibrillation when a defibrillator was not available. One died of ventricular fibrillation after the septum was cut during the procedure. An occasional dog developed ventricular fibrillation on the table but was defibrillated and survived without untoward effects.

Postoperative Complications.—Six dogs died postoperatively. One died of rupture through the incision in the aorta; one died from massive infestation with heart worms which occluded the pulmonary artery; another dog died suddenly 12 days postoperatively without pre-existing illness and was not autopsied because of laboratory error; another died from pulmonary edema on the third day. Two dogs with empyema due to retained sponges died, one 5 days postoperatively with nonbacterial vegetative endocarditis and the other 2 weeks postoperatively with massive bacterial vegetations on the aortic cusp. There were 2 other instances of empyema, but both dogs responded to therapy. An incidental finding in one dog was the presence of blastomycotic lesions in the right lung. This did not appear to bother the dog in his subsequent course and there was no evidence of dissemination at autopsy.

Technique of Follow-up.—Dogs were examined daily for a period of 7 to 10 days postoperatively and then at frequent intervals throughout the remainder of their course. Rectal temperatures were recorded. Murmurs were observed, along with weights, temperature, and clinical status, and were recorded at each examination.

Strain of Staphylococcus aureus.—The Harmon strain of *Staphylococcus aureus hemolyticus* was recovered from a human case of acute bacterial endocarditis. The minimal inhibiting concentration of penicillin is 31 μg per milliliter and of tetracycline 0.25 μg. Its unusual bacteriophage type (6/7/42E/47/54/75/971*) makes this strain particularly useful because of the ease of identifying it when recovered from blood or tissues. One to four representative colonies from each blood or tissue culture were typed, and the sensitivity to penicillin and tetracycline was determined by a twofold serial dilution method. Several hundred strains have been typed during the course of the study so far.

Production of Endocarditis.—At varying periods of time after the operation, 1 ml. of a 4½ hour culture of the Harmon strain of *Staphylococcus aureus* was injected intravenously. Blood cultures were obtained before inoculation and at daily or nearly daily intervals after inoculation. Duplicate pour plates of 1 ml. of blood were made, and approximately 1.0 ml. was injected into a flask of broth. When large numbers of organisms were present, quantitative counts were made by obtaining 0.2 ml. of broth in a tuberculin syringe to which was added 0.8 ml. of broth.

Autopsies on infected animals were performed with strict asepsis. Some were sacrificed by exsanguination under Nembutal anesthesia. In all cases the heart was removed in toto by ligating all its vessels and placing it in a sterile beaker. Sections were taken from the heart valve and left ventricle, lung, liver, spleen, kidney, adrenals, and other tissues as seemed to be indicated by the gross pathologic findings. Quantitative cultures of tissue aliquots were made following grinding of the tissue with sand and broth.

RESULTS

Natural History of Dogs Developing Bacterial Endocarditis Following Intravenous Inoculation of Staphylococcus aureus.—The intravenous injection of 10^7 to 10^8 staphylococcal cells was followed by acute endocarditis in 11 or 55 per cent of 20 dogs. Clinically the 11 dogs became ill 2 to 6 days after the inoculation, although blood cultures were positive during the latent period between inoculation and clinical illness. The number of colonies per milliliter of blood frequently remained below 10 during the first few days after inoculation, but later rose to hundreds and sometimes thousands. This quantitative increase in bacteremia usually coincided with the development of the clinical phase of the dog's illness. After the latent period they did not eat and lay listlessly in their cages. In a minority of animals petechiae appeared in the conjunctivae and buccal mucous membranes. The aortic diastolic murmur usually did not change perceptibly in quality. The rectal temperature rose to between 102 and 106 degrees in every dog but one. Table I presents the clinical chart of Dog 57-928, illustrating these points.

The time interval between the operation upon the aortic valve and the intravenous inoculation of staphylococci was 17, 23, 30, 30, 43, and 73 days in the 6 untreated dogs in this series which developed endocarditis. In 5 treated dogs, to be reported in a separate paper, the interval between operation and inoculation varied between 24 and 42 days.

*We are indebted to Dr. J. B. Wilson, University of Wisconsin, Dr. R. E. O. Williams, Central Public Health Laboratory, London, England, and Dr. George Gee Jackson, University of Chicago, for the bacteriophage identification of this strain.

In order to obtain accurate measurements of the numbers of staphylococci present in the aortic valve and in selected tissues at autopsy, every effort was made to observe the clinical course of the animal carefully in order to sacrifice it when its life expectancy seemed limited to 24 hours or less. In a few instances dogs that looked as though they would live another 24 hours died during the night.

TABLE I. CLINICAL COURSE OF A DOG WITH STAPHYLOCOCCAL AORTIC VALVE ENDOCARDITIS

OBSERVATION	DAY								
	1	2	4	5	6	7	8	9	12
Temperature (F.)	102.3°	103.7°	103.5°	103.6°	103.5°	103.2°	105.5°	105.5°	104.5°
Clinical condition	Good	Good	Good	Good	Good	Good	Sick	Worse	Sacrificed
Blood cultures									
Broth	0	+	+	+	+	+	+	+	+
Colonies on plate	0	0	3	4	4	230	104	2,150	3,680 1,500 (Day 11)

Six such untreated dogs were sacrificed. Perforations were present in one aortic cusp of 5 of the dogs and in 2 cusps of the sixth. There was gross evidence of roughening at the edges of these perforations, but in only 2 instances were gross vegetations found. However, microscopic sections revealed striking evidence of acute endocarditis, with infiltration of polymorphonuclear leukocytes, deposition of fibrin, and huge masses of gram-positive cocci. Such a lesion is shown in Fig. 1. The photomicrographs marked *a, c,* and *d* illustrate these findings and demonstrate the distribution of staphylococci in the acute lesions. It is readily seen that some of the bacteria are imbedded deep within the vegetations but others, as shown in the high-power enlargement in the upper right corner, were found at the edge of the lesion, adjacent to the blood stream. The polymorphonuclear infiltrate was restricted to the vegetation itself and did not involve the surrounding valve tissue except in a few instances when the mitral or tricuspid valves were affected. An analysis of the aortic valve sections showed no correlation between the degree of polymorphonuclear reaction and the time interval between the inoculation of staphylococci and death.

In several of the hearts there was a cobblestone-like appearance to the undersurface of the tricuspid valve, and occasionally some redness and hemorrhage in its base and upon the mitral cusp were present. Gross vegetations were not found on any other valve. However, the mitral valve of Dog 57-69 was the seat of acute endocarditis with fibrin, polymorphonuclear infiltration and many gram-positive cocci. Two other mitral valves, in Dog 57-28 and Dog 57-111, microscopically displayed superficial necrosis and edema with mild to moderate polymorphonuclear infiltration, but no visible staphylococci. A similar microscopic reaction was found in the tricuspid valve of Dogs 57-928 and 57-871.

In most, but not all, of the dogs petechial hemorrhages were found under the epicardium and in the mediastinum. In one animal a gross myocardial abscess was visible.

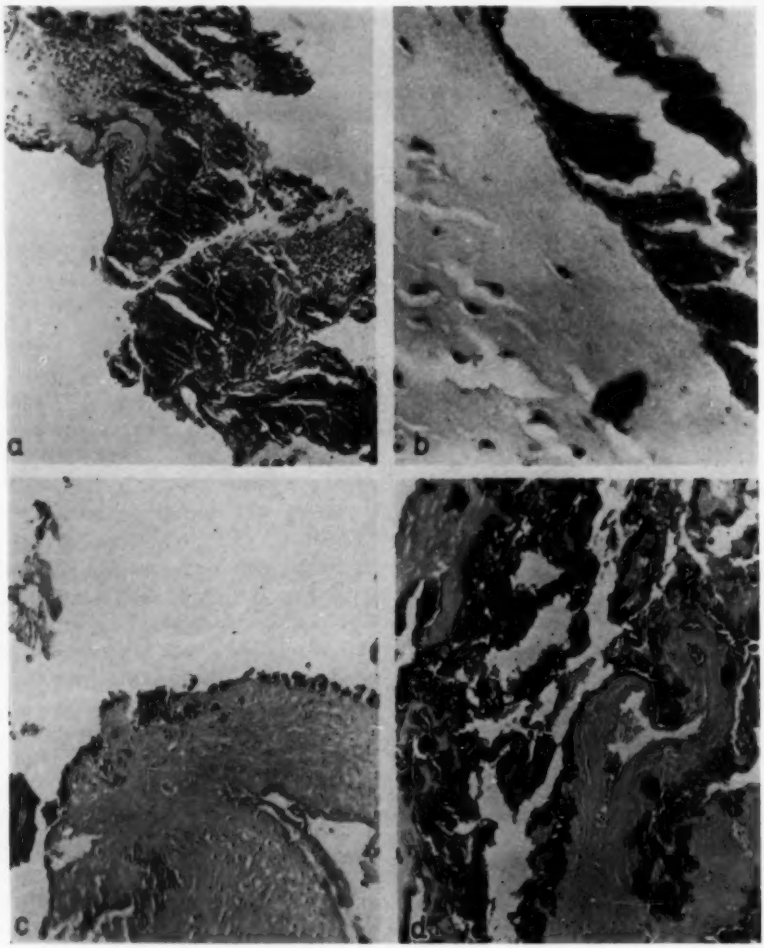

Fig. 1.—Microscopic sections of aortic valve lesions of 4 dogs with acute staphylococcal endocarditis. (*a*, *c*, and *d*, Magnification ×160; reduced 2/7. *b*, Magnification ×650; reduced 2/7.)

Outside the heart the most striking lesions were seen in the kidney. Most of the kidneys revealed small red flecks on the surface, extending into the medulla, and a few displayed gross renal infarcts. A few splenic infarcts were seen, but the livers were grossly normal. Sometimes small infarcts were seen in the lungs but they, too, more often appeared grossly normal.

Quantitative cultures of the involved valves and other tissues proved to be of great interest. The data are presented in Table II. It is seen at a glance that the aortic cusp yielded 10^9 to 10^{11} staphylococci per gram, despite the usual absence of gross vegetation. In contrast, the left ventricle, kidneys, liver, spleen, and lungs yielded far fewer bacteria, the counts ranging from 10^3 to 10^6, with one kidney count of 10^7.

TABLE II. NUMBER OF STAPHYLOCOCCI PER GRAM OF TISSUE IN DOGS WITH GROSS OR MICROSCOPIC ACUTE ENDOCARDITIS

DOG	AORTIC CUSP	LEFT VENTRICLE	KIDNEY	LIVER	SPLEEN	LUNG	TRICUSPID VALVE	MITRAL VALVE
57-111	2×10^{10}	2×10^6	2×10^7	4×10^5	2×10^5	2×10^6	—	—
57-871	—	4×10^6	9×10^5	3×10^5	6×10^5	—	2×10^7	—
57-928	1×10^{11}	1×10^6	7×10^5	9×10^4	5×10^5	5×10^5	—	8×10^7
57-86	2×10^9	2×10^6	3×10^6	3×10^5	2×10^5	1×10^5	—	—
57-920	2×10^{10}	8×10^4	4×10^4	4×10^4	3×10^4	3×10^5		
57-69	2×10^{10}	1×10^5	6×10^5	2×10^5	4×10^5	3×10^5	—	2×10^{10}

Figures represent staphylococci per gram of tissue. Dash (—) indicates that no determination was made.

One case of spontaneous vegetative endocarditis occurred in an uninoculated dog. This animal developed a febrile illness one week after the operation and was sacrificed on the fifteenth postoperative day. At autopsy he was found to have a sponge in the right pleural cavity and massive empyema from which *Staphylococcus aureus* was cultured. A huge vegetation was found on the perforation in the operated aortic cusp, and a "kissing vegetation" on one of the other cusps. Culture of the vegetation yielded 2×10^7 staphylococci per gram, while other tissues contained 10^2 to 10^4 staphylococci per gram. However, these staphylococci did not possess the characteristics of the Harmon strain.

Dogs Which Failed to Develop Endocarditis Following the Intravenous Injection of Staphylococcus aureus.—Nine dogs failed to develop endocarditis despite the intravenous injection of the Harmon strain of *Staphylococcus aureus.*

Four dogs had no murmurs at the time of injection. One (57-921) was inoculated 29 days after the operation and sacrificed 6 days later. Four blood cultures during life were sterile. At autopsy the right coronary cusp was found to have a 2 by 2 mm. defect at the base, but no gross or microscopic evidence of endocarditis was present. Liver, spleen, and kidneys were grossly normal. Thirteen colonies of the Harmon staphylococcus were recovered per gram of kidney tissue but none from the other tissues. Dog 57-180 was inoculated 27 days after the operation, reinoculated 15 days later and sacrificed 6 days after the second inoculation. Eleven blood cultures during life were sterile, although the tissues at autopsy yielded small numbers of Harmon staphylococci. Two hundred and twenty-two colonies were recovered per gram of kidney tissue, 43 per gram of heart muscle, 24 per gram of spleen, 8 per gram of liver, but none from the lungs or from the aortic cusp. Grossly a defect was found in the right coronary cusp measuring 2 mm. in diameter with thickened rough and hard

edges, resembling a doughnut. Numerous small areas of hemorrhage were found in the medulla of the kidney, along with occasional collections of round cells. The lungs were the seat of diffuse pneumonia; the liver, spleen, and left ventricle were normal. Dog 57-160 was likewise given 2 inoculations of staphylococci, 51 and 56 days postoperatively. All the blood cultures during life were negative. When the animal was sacrificed 11 days after the second inoculation a doughnutlike lesion was found in one of the aortic cusps and a few petechial hemorrhages in the medulla of one kidney. The latter also showed microscopic evidence of acute inflammation and hemorrhagic infarction. The aortic cusp microscopically was thickened with collagen and loose connective tissue, but no bacteria nor any evidence of acute inflammation were encountered. Despite these negative findings, Harmon staphylococci were found in some of the tissues, as follows: aortic cusp 2.7×10^4; left ventricle 1×10^2; kidney 4.4×10^4; and lungs 1×10^4.

Dog 57-908 received inoculations of staphylococci 110 and 142 days after the aortic valve operation. The only positive blood cultures were on Days 1 and 10 following the second inoculation—1 colony per milliliter of blood in each culture. When the animal was sacrificed 14 days after the second inoculation the heart, lungs, kidneys, spleen, and liver were all sterile. One of the aortic cusps of this animal also displayed the doughnut lesion and microscopically its appearance resembled the others. The remaining tissues were normal.

Five dogs failed to develop endocarditis despite the presence of good aortic diastolic murmurs at the time of inoculation with staphylococci. Dog 57-224 was inoculated 24 days after the operation, Dog 57-120, 90 days, Dog 57-12, 118 days, Dog 57-217, 27 and 34 days, and Dog 57-179, 35, 48 and 56 days. None became clinically ill. None of the blood cultures of Dog 57-120 were positive. Blood cultures drawn during the first 2 or 3 days from Dogs 57-12, 57-179, and 57-224 yielded a maximum of 4 colonies, following which they were consistently negative. Dog 57-217 had a colony count of 84 twenty-four hours after inoculation but after that no more than 5 colonies were obtained on any day during the first week; after the first week cultures became sterile. These 5 dogs remained well and were sacrificed 5 months after the last inoculation of staphylococci. In every instance there was a doughnut-like lesion at the site of operation but the remainder of the tissues were grossly and microscopically normal.

Blood pressure records were made in 4 dogs via a needle in the femoral artery that connected with the 4-channel Sanborn polygraph. These readings were made under Nembutal anesthesia. The pressures, recorded 60 to 140 days postoperatively, were 200/105, 190/105, 200/105, and 250/105, respectively. One of these dogs had defects in each of 2 aortic cusps, the remaining 3 in one cusp only. Thus, it is seen that these pulse pressure readings could not be correlated with the amount of aortic insufficiency found at autopsy.

DISCUSSION

Our experience has confirmed the demonstration of Highman, Roshe, and Altland[12] that dogs given injections of *Staphylococcus aureus* following the artificial production of aortic insufficiency will develop fatal acute ulcerative endocarditis. Technical problems arose in the surgical procedure of preparing the aortic insufficiency dogs, but these difficulties did not constitute a major drawback. A few dogs were lost as a result of ventricular fibrillation, of postoperative complications and of postoperative rupture of the suture in the wall of the aorta.

In general, the induction of endocarditis was most successful when the operation had resulted in a loud aortic diastolic murmur; under these conditions successful inoculations occurred in 11 out of 16 dogs, or 68 per cent. Also, in our experience, dogs inoculated within the first 6 weeks of the postoperative period were more likely to develop endocarditis following a single inoculation than were dogs injected after this period. Only a few of our animals received a second inoculation of staphylococci and a few a third, but none more than 3. In contrast, Highman and associates[12] gave 4 to 13 intravenous injections of staphylococci, at unstated intervals, and were able to produce endocarditis in 100 per cent of 7 dogs. Three of these dogs were evidently first inoculated more than 3 months after the operation upon the aortic valve. It seems possible that had we given additional inoculations to animals which failed to "take" after 1 or 2 or 3 injections the incidence of endocarditis in our dogs may have been higher. It seems possible, also, that the histologic characteristics of the tissue surrounding the perforation in the valve cusps, as healing progressed after the operation, might have a bearing on the dog's susceptibility to bacterial endocarditis. The healing lesion grossly resembled a doughnut and microscopically consisted of collagen tissue beginning to be organized. It seemed likely that such a lesion would resist the implantation of staphylococci better than would a fresh injury to the valve. Perhaps larger numbers of staphylococci could overwhelm the inherent resistance of the healing lesion.

The clinical course of the dogs was interesting and paralleled closely that seen in many human cases of acute staphylococcal endocarditis. For several days the dogs remained essentially well despite small numbers of staphylococci found in the blood cultures. However, there appeared to be a point at which the animals' resistance suddenly gave way, followed by rapidly progressing clinical infection and enormous increases in the numbers of staphylococci found in the blood stream. The petechiae seen in the conjunctivae of a few of the animals were identical with those seen in human patients.

As far as we know these studies provide the first quantitative demonstration of the numbers of staphylococci present in the valves of subjects with acute endocarditis. The lesions in the aortic valve contained from 1,000 to 10 million times as many staphylococci as were found per gram in various other tissues. Actually this is not surprising when one notes the huge masses of organisms visible in the sections of the valves themselves. The distribution of staphylococci in the valve sections also provides a clue to the numbers of bacteria in

the blood cultures, to the dissemination of staphylococci in the tissues, and to the variability in the results of therapy observed in human beings. For example, the staphylococci are sometimes found bordering the edge of the valve, as observed by Highman and co-workers.[12] Other times the masses of organisms are enmeshed in fibrin and not in direct contact with the blood stream. Sometimes the staphylococci are still deeper in the valve tissue. In one dog (to be reported in another paper), where the animal's course was prolonged for months by treatment, both healing and acute inflammation, with many staphylococci, were present in the same lesion. In this animal ultimate healing was prevented by the development of antibiotic resistant staphylococci.

Histologically the valvular lesions were typical of a rapidly moving infectious process. The intensity of the polymorphonuclear response varied from animal to animal, but heavy fibrin deposition was present in all of the valves. Another counterpart with staphylococcal infections in other species, such as man and mouse,[14] was the rather uniform involvement of the kidneys but much less common involvement of spleen and liver. In one dog an abscess appeared in the myocardium, an occasional finding also in the human disease.

SUMMARY

Acute staphylococcal endocarditis has been produced in dogs following operative perforation of the aortic valve and subsequent inoculation of a virulent strain of *Staphylococcus aureus*. Ventricular fibrillation occasionally caused trouble at the operating table, and sometimes postoperative infections and aortic rupture occurred. However, the operative technique is a satisfactory one, and the incidence of endocarditis following a single inoculation of approximately 10^7 staphylococcal cells was high. The course of dogs with acute staphylococcal endocarditis resembled that seen in human beings and was rapidly fatal within a few days to 2 weeks.

Histologic study of the infected valves showed huge masses of staphylococci usually enmeshed in fibrin with varying degrees of polymorphonuclear reaction. Quantitative cultures of the valves yielded 10^9 to 10^{11} staphylococci per gram of tissue, as contrasted with 10^3 to 10^6 or 10^7 in other tissues.

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LABORATORY METHODS

THE MEASUREMENT OF AMMONIA IN PLASMA AND BLOOD

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EXAMINATION of the literature on the effects of ammonium salts, or other agents which give rise to ammonia, in both animals and human beings,[1-10] discloses a striking correlation between the administration of such compounds and the appearance of a group of symptoms indistinguishable from those of hepatic coma. In contrast, reported measurements of blood ammonia have not always given consistent correlations with the symptomatology of hepatic coma.[6-8, 10-12] We have proposed the hypothesis that the free ammonia is the significant factor in the symptomatology of hepatic coma.[13, 14] In attempts to test this hypothesis, it became apparent that much of the confusion in this field was due to the different methods of measuring ammonia in body fluids and that a re-evaluation of these methods was needed.

The measurement of blood ammonia has generally involved alkalinization followed by aeration, vacuum distillation, or diffusion of ammonia into an acid reservoir. Nencki and Zaleski[15] were the first to recognize that the ammonia obtained from blood increased with the amount of alkali added to the sample. Because of this most of the values for blood ammonia obtained up to 1924 are far too high. In 1924, Parnas and Heller[16] reviewed the work up to that time and presented a new vacuum-steam distillation method which was superior to the previous methods. They demonstrated that (1) the blood ammonia content increases when the blood is allowed to stand after drawing. This process is fluoride insensitive, independent of P_{O_2}, and increases with the temperature as well as with pH up to a pH of 8.1. When the cells and plasma were separated, ammonia formation occurred in both but was much faster in the cells. (2) The ammonia content of a sample increased during the preparation of a protein-free tungstic acid filtrate.

In 1933, Conway and Byrne[17] presented their microdiffusion method for determining ammonia. Studies on ammonia formation in blood and on diffusion kinetics[18, 19] showed the following: (1) Ammonia is formed in oxalated blood on standing. Conway found this process to occur in 3 phases (α, β, and γ),

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the first of which occurred within the first 5 minutes after the blood was shed into a vessel under carbon dioxide. Calkins[20] has repeated Conway's measurements on blood drawn anaerobically into a syringe and has not found any ammonia formation until 20 minutes after withdrawal of the blood. Of a large number of compounds tested by Conway, only adenosine, the adenylic acids, adenosinetriphosphoric acid, and thymus nucleic acid gave rise to ammonia on addition to blood. (2) With an ammonium salt standard, the diffusion of ammonia into the acid reservoir follows an exponential curve, $X = Q\,[1 - e^{-kt}]$, where X is the amount diffused in time t, Q is the total ammonia initially present, and k is the diffusion constant for the system. (3) With a sample of blood there is an exponential diffusion curve plus a production of ammonia due to the action of the alkali on the blood (1 ml. blood plus 1 ml. saturated K_2CO_3). The ammonia formation is linear in time for the first few hours with an average rate of ammonia formation of 0.53 μg of NH_3–N per hour per milliliter of blood. (4) The diffusion constants for ammonia standards and for blood differ.

In 1951, Seligson and Seligson[21] introduced a modified diffusion method using penicillin bottles for diffusion flasks. This method was readily adaptable to routine clinical use. Seligson has been concerned about the ammonia formation by the alkali and has recently published a modification of his older method, using a mixture of K_2CO_3 and $KHCO_3$ for alkalinization of blood. This gives a smaller rate of ammonia formation. Seligson's methods and modifications thereof are widely used. However, no complete studies of diffusion kinetics and ammonia formation have been made to check whether the findings of Conway[17, 18] are applicable to this system. The present study is intended to do this.

The determination of ammonia in biologic fluids can be considered under two separate problems, namely, (1) How can one measure the ammonia in a sample knowing the diffusion kinetics and knowing that there will be a production of ammonia during the determination? (2) What sampling and handling procedure is required to assure that there will be no change in ammonia from the time of drawing the sample until it is used for the ammonia measurement?

MATERIALS AND METHODS

Apparatus.—The method of Seligson and Seligson[21] was used for a number of studies with 3 different sized diffusion bottles. These were: (1) Small—penicillin bottles as used by Seligson and Seligson. (2) Medium—Varidase bottles, 6.3 cm. high, 3.6 cm. in diameter, 36 ml. in volume. (3) Large—10.4 cm. high, 5.1 cm. in diameter, 120 ml. in volume. Studies with the new technique of Seligson and Hirahara[22] followed their methods. These will be referred to as the Seligson K_2CO_3 method and the Seligson K_2CO_3–$KHCO_3$ method. Two rotators were used, one set at 13 r.p.m., the other at 54 r.p.m. The Coleman Model 14 spectrophotometer was used for spectrophotometry of the color developed with Nessler's reagent at a wave length of 405 mμ. Coleman cuvettes, 12 by 75 mm., containing 2 ml. of Nessler's reagent were used. The receiving rods in No. 00 rubber stoppers fitted these cuvettes, as well as all of the diffusion bottles.

Reagents.—1. $K_2CO_3 \cdot 1.5\ H_2O$ (Baker and Adamson, A.C.S.). The saturated solution was boiled and stored in stoppered Pyrex bottles. For the Seligson K_2CO_3 method a scoop was used which delivered 2.8 Gm. into the diffusion bottles. A diffusion study on standards should be run with each new batch of K_2CO_3 to determine the blank value for the reagent and to check the diffusion constant.

2. $KHCO_3$ (Merck Reagent). The mixture for the Seligson K_2CO_3–$KHCO_3$ method was made up as directed by Seligson and Hirahara.[22]

3. H_2SO_4 1 N (Acculute, Anachemia Chemicals, Ltd.).

4. Nessler's Reagent (APHA, Fisher Scientific Company). Dilute 1:10 for use.

5. Stock ammonia standard, $(NH_4)_2SO_4$ solution, 100 μg NH_3–N per milliliter. This was diluted appropriately for working standards in the range 0.5 to 10 μg per milliliter.

6. Distilled water (single distilled).

Procedure.—The procedure for diffusing the sample has been described.[21, 22] The acid-dipped receiving rods were transferred from the diffusion bottles to the Coleman cuvettes. All of the cuvettes, including the blank, were inverted at the same time to wash the receiving rod with the Nessler's solution. They were allowed to stand 10 minutes for color development before reading.

Handling of Blood and Plasma.—Except for experiments in which this procedure was varied to study the methods of handling blood and plasma, the following standard technique was used. Blood was drawn aseptically and anaerobically into a heparinized syringe and iced immediately. Plasma was obtained by centrifuging the blood in 50 ml. syringes cut down to allow centrifugation in a size 2 International Centrifuge. The plasma was delivered through rubber adapters from the syringe into the volumetric pipettes.

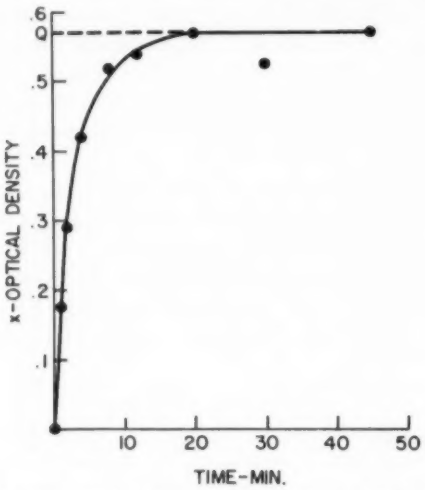

Fig. 1.—Diffusion kinetics for the Seligson K_2CO_3 method.

RESULTS

I. *Diffusion Kinetics of Standards.*—Figs. 1 and 2 show typical diffusion curves using the Seligson K_2CO_3 and K_2CO_3–$KHCO_3$ methods, respectively. The plot of $\frac{Q}{Q-X}$ on a semilog scale in Fig. 3 shows that the diffusion process closely fits the exponential curve $X = Q\ [1-10^{-kt}]$, where Q is the total ammonia in the diffusion bottle, X is the amount which has diffused to the receiving

rod in time t, and k is the diffusion constant for the system. Table I summarizes diffusion studies on the effect of varying the size of the diffusion bottle and the volumes of the liquid and gas phases on the diffusion constants. Included is the time for 98 per cent of the ammonia ($t_{98\%}$) to diffuse over to the

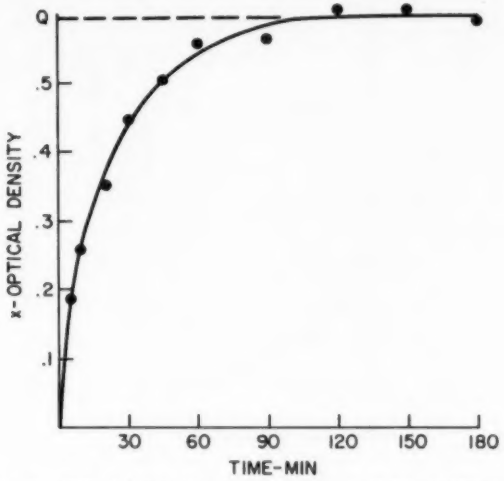

Fig. 2.—Diffusion kinetics for the Seligson K_2CO_3–$KHCO_3$ method.

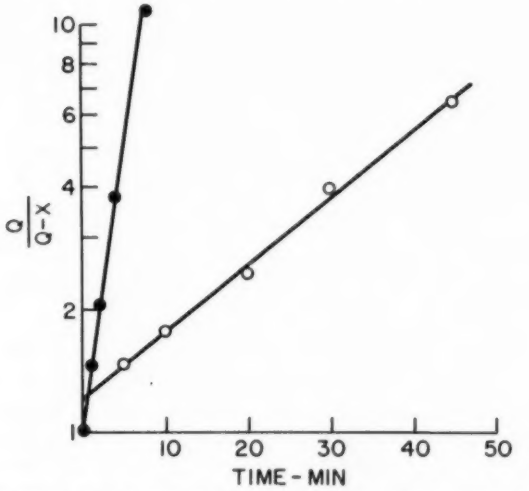

Fig. 3.—Semilog plot of the data in Figs. 1 and 2. ● = Seligson K_2CO_3 method. ○ = Seligson K_2CO_3–$KHCO_3$ method.

receiving rod. It is obvious that mixing in the liquid phase and diffusion from liquid phase to gas phase is of major importance in determining the diffusion constant for a system. Diffusion in the gas phase is apparently so fast that it

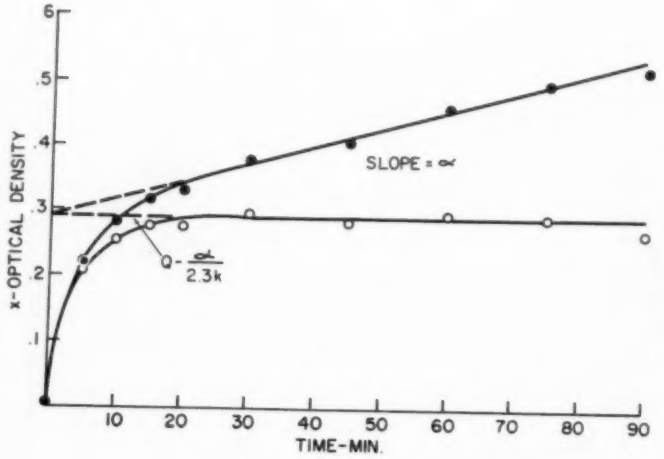

Fig. 4.

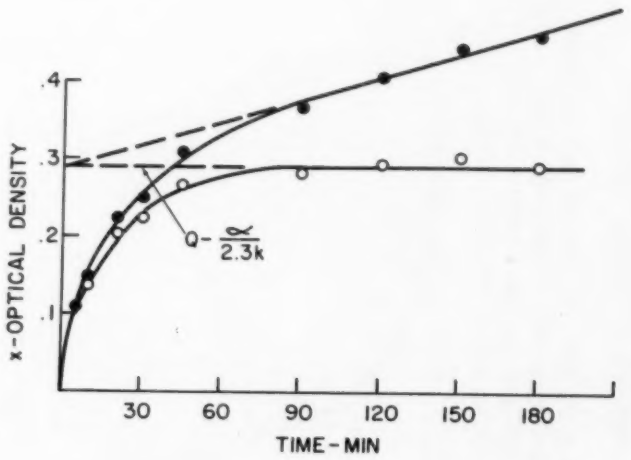

Fig. 5.

Fig. 4.—Diffusion curve for dog plasma; Seligson K_2CO_3 method. Experimental data (●). Curve obtained by subtracting at from the experimental data (○).

Fig. 5.—Diffusion curve for dog plasma; Seligson K_2CO_3-$KHCO_3$ method. ● = Experimental data. ○ = Curve obtained by subtracting at.

plays a minor role. A basic principle governing the use of a system of this type for analytic purposes is to avoid the time dependent portion of the diffusion curve and make all determinations at diffusion times greater than $t_{98\%}$ or even $t_{99.5\%}$. This has to be modified when the diffusion kinetics of plasma is considered. By this criterion the Seligson K_2CO_3 method is superior because of the short diffusion time required. Most of the remaining work was done with the Seligson K_2CO_3 method or the K_2CO_3–$KHCO_3$ method.

TABLE I. SUMMARY OF STUDIES OF DIFFUSION KINETICS OF AMMONIA STANDARDS

DIFFUSION BOTTLE	VOL. OF STANDARD ADDED (ML.)	K_2CO_3 ADDED	ROTATOR R.P.M.	IS DIFFUSION EXPONENTIAL?	k (HR.)$^{-1}$	$t_{98\%}$ (HR.)	COMMENTS
Small	1	1 ml. Sat.	13	Yes	1.7	1.0	
	1	2 ml. Sat.	13	Yes	1.3	1.3	
	2	2 ml. Sat.	13	Yes	0.57	3.0	
Medium	1	1 ml. Sat.	13	Yes	0.9	1.9	
	2	2 ml. Sat.	13	Yes	0.74	2.3	
	3	3 ml. Sat.	13	Yes	0.55	3.1	
Large	1	1 ml. Sat.	13	Yes	0.86	2.0	
	2	2 ml. Sat.	13	Yes	0.62	2.7	
	5	5 ml. Sat.	13	Yes	0.42	4.0	
Seligson and Hirahara[22]	2	2 ml. Sat.	54	Yes	1.07	1.6	3 steel rods in diffusion bottle
	2	2.8 Gm.	54	Yes	8.2	0.21	Seligson K_2CO_3 method
	2	2.8 Gm.	54	Yes	7.5	0.23	Seligson K_2CO_3 method
	2	K_2CO_3-$KHCO_3$	54	Yes	0.85	2.0	Seligson K_2CO_3-$KHCO_3$ method
	2	K_2CO_3-$KHCO_3$	54	Yes	0.97	1.75	Seligson K_2CO_3-$KHCO_3$ method

II. *Diffusion Kinetics of Plasma and Blood.*—Fig. 4 shows the diffusion curve obtained on a sample of dog plasma from stored blood, using the Seligson K_2CO_3 method. Fig. 5 gives the corresponding curves obtained by the Seligson K_2CO_3–$KHCO_3$ method. In agreement with Conway's findings the formation of ammonia is linear in time. If the ammonia formed (αt) is subtracted from the experimental points, one obtains an exponential diffusion curve from which the diffusion constant for the system can be determined. If one assumes exponential diffusion kinetics and a constant rate of ammonia formation, it can be shown mathematically that the diffusion curve will be $X = \left[Q - \frac{\alpha}{2.3k}\right][1 - 10^{-kt}] + \alpha t$, where Q is the total ammonia present in the diffusion flask at $t = 0$, α is the rate of ammonia formation, k is the diffusion

constant, and X is the amount of ammonia which has diffused over to the receiving rod in time t. If the ammonia formation (αt) is subtracted, an exponential diffusion curve is obtained; however, the plateau is at $Q - \frac{\alpha}{2.3k}$ not Q. This is the same result as that obtained by Conway[18] in his microdiffusion system. Fig. 6 shows diffusion curves on normal human venous blood and the plasma therefrom, using the Seligson K_2CO_3 method. Table II summarizes diffusion studies on dog plasma with the Seligson K_2CO_3 method and the Seligson K_2CO_3–$KHCO_3$ method. Included in the table are values of α/k. In comparing a number of diffusion systems, it is obvious that the best one is the one which has the least ammonia formation during comparable diffusion times.

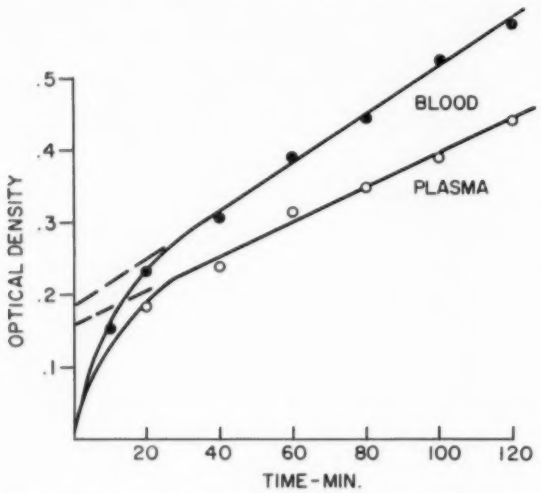

Fig. 6.—Diffusion curves of human blood and the plasma therefrom; Seligson K_2CO_3 method.

Thus the best system is the one which has a minimum value of α/k. By this criterion, the Seligson K_2CO_3 method is superior to the Seligson K_2CO_3–$KHCO_3$ method.

TABLE II. DIFFUSION STUDIES ON DOG PLASMA

METHOD	α = RATE OF NH_3 FORMATION μg NH_3-N PER. HR.	k = DIFFUSION CONSTANT $(HR.)^{-1}$	$t_{98\%}$ (HR.)	α/k
Seligson K_2CO_3	1.9	5.2	0.33	0.37
Seligson K_2CO_3	2.0	5.0	0.34	0.40
Seligson K_2CO_3-$KHCO_3$	0.62	1.1	1.5	0.56
Seligson K_2CO_3-$KHCO_3$	0.65	0.7	2.4	0.93

III. *The Accurate Measurement of Ammonia in Plasma and Blood.*—In light of the above findings we have used the following method for investigations on plasma and blood ammonia. The Seligson K_2CO_3 method has been used throughout.

Diffusion of standards: A complete set of standards is diffused daily. The diffusion time used is 30 minutes which is longer than the $t_{99.5\%}$. The error of the method was determined by running duplicate sets of standards on successive days. The standard curve, shown in Fig. 7, is linear up to 6 μg NH_3–N. The standard error is ± 0.1 μg NH_3–N.

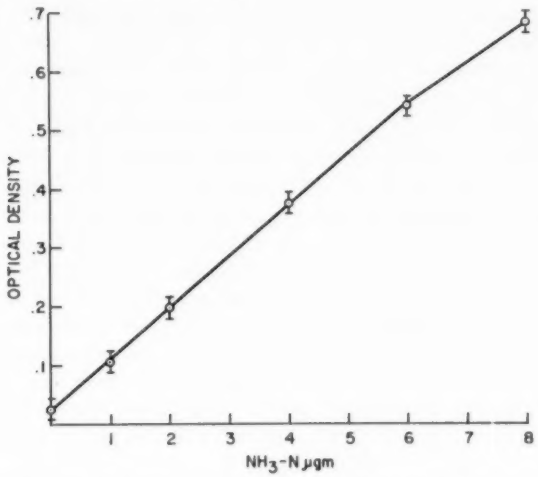

Fig. 7.—Standard curve, Seligson K_2CO_3 method. The brackets represent the 95 per cent confidence intervals.

Diffusion of plasma or blood: Samples are diffused for 4 different times on the linear portion of the diffusion curve, usually 30, 45, 60, and 75 minutes. The best straight line is drawn through the 4 points and extrapolated back to zero time to give $\left[Q - \frac{\alpha}{2.3k}\right]$. The rate of ammonia formation (α) is given by the slope of the line. Using reasonably careful technique all 4 points fall closely on a straight line. Occasionally one point is markedly out of line with the other 3, this is disregarded and only the 3 points are used. Duplicate runs were made on 10 samples of plasma and the standard errors of the duplicates were determined. These were ± 0.18 μg for the extrapolated value $\left[Q - \frac{\alpha}{2.3k}\right]$ and ± 0.16 μg per hour for α, for 2 ml. samples. Thus the standard errors are less than ± 0.1 μg per milliliter for $\left[Q - \frac{\alpha}{2.3k}\right]$ and less than ± 0.1 μg per hour per milliliter for α. The correction factor $\alpha/2.3k$ is of the same order of magnitude as the error; however, it should be calculated to determine Q since it is a one-sided bias and not a random error. This method is relatively insensitive to variation in k because the diffusion constant appears only in the correction factor $\alpha/2.3k$. We have used an average value for k for human and dog plasma of 5 $(hr.)^{-1}$.

This method is superior to any of the methods which obtain only one point on the diffusion curve because the rate of ammonia formation in the system is

measured and corrected for. However, the work involved and the size of sample required make it impractical for routine clinical use. The rates of ammonia formation in human plasma have fallen in the range of 0.61 to 1.6 μg per hour per milliliter by our method. Thus, no simple correction factor can be used to correct values obtained from a single diffusion time. Nonetheless, the single diffusion time determinations can still be useful for routine purposes once it is recognized that the values obtained are approximate and contain a variable contribution due to ammonia formation in the diffusion system and an inherent error due to different diffusion constants for the standards and plasmas.

IV. *The Formation of Ammonia in Alkalinized Plasma.*—It has generally been considered that the ammonia formed during the diffusion of blood comes from labile amides.[22] We tested a number of compounds at levels equal to or higher than the maxima found in normal plasma. Glutamine, asparagine, arginine, glycine, adenosine, creatine, guanidine, and urea did not form any ammonia in our system. Ethanolamine at a level of 3.3 mM. per liter formed ammonia at a constant rate of 0.4 μg per hour per milliliter. The ethanolamine formed by saponification of phosphatides in plasma could account for only a minor portion of the ammonia formed. Further studies on plasma and plasma fractions are summarized in Table III. Most of the ammonia formed comes from the nondialyzable fraction. It is known that serine and threonine are unstable to alkali when in peptide linkage[23, 24] and give rise to ammonia during alkaline hydrolysis of proteins. This probably accounts for the major part of the ammonia formed in the diffusion of plasma and blood.

TABLE III. AMMONIA FORMATION DURING DIFFUSION OF PLASMA AND PLASMA FRACTIONS

SAMPLE	AMMONIA FORMATION (α − μg/ML./HR.) ORIGINAL PLASMA	DIALYZED PLASMA	PLASMA ULTRAFILTRATE	COMMENTS
Human plasma	1.33	0.90	—	Some protein precipitate formed during dialysis. Removed by centrifugation
Dog plasma	0.96	0.97	—	Faint protein precipitate formed during dialysis. Removed by centrifugation
Dog plasma	0.79	0.67	0.28	—

V. *Handling of Blood and Plasma.*—Assuming one can measure blood or plasma ammonia accurately, there still remains the problem of how to handle the blood to prevent the enzymatic formation of ammonia described by Parnas and Heller[16] and Conway and Cooke.[19] Considerable information on this is in the literature. Since the data available were obtained by single diffusion time methods it was deemed necessary to repeat most of these experiments with our accurate method. The results and pertinent references are as follows.

Sampling: The blood should be obtained anaerobically and without stasis. Since the distribution of ammonia between cells and plasma is dependent on pH,[14] it is essential that the blood be drawn anaerobically. This is also necessary to prevent the increase in pH from CO_2 loss which results in increased

ammonia formation by enzymatic processes.[16, 19] It was found that clenching and opening the fist for a brief period led to a rise in ammonia in blood from the antecubital vein, corroborating the findings of Kalk and Bonis.[25]

Handling: If freshly drawn blood is left at room temperature, there is little rise in ammonia up to 15 to 20 minutes; thereafter there is an increase. This corroborates Calkins'[20] findings. If the blood is iced immediately it can generally be kept for 2 hours before any appreciable increase in ammonia occurs.

Separation of plasma: No significant difference was found between the ammonia content of plasma separated from the blood cells at room temperature or at 5 to 15° C., provided the total handling time at room temperature was less than 20 minutes. Thus, the distribution of ammonia between plasma and red cells is at least not markedly dependent on temperature.

VI. *Blood and Plasma Ammonia.*—Using the techniques described we have determined venous blood and plasma ammonias on a number of young adults. These values, including some simultaneous plasma and whole blood values are shown in Table IV. The plasma levels show less variation than the blood levels and the rate of ammonia production is generally higher in blood than in plasma.

TABLE IV. AMMONIA LEVELS IN VENOUS BLOOD AND PLASMA FROM NORMAL YOUNG ADULTS

	PLASMA		BLOOD	
SUBJECT	$\underline{Q}$ $\overline{NH_3}$–N μg/ML.	α $\overline{NH_3}$–N μg/HR./ML.	$\underline{Q}$ $\overline{NH_3}$–N μg/ML.	α $\overline{NH_3}$–N μg/HR./ML.
J. P.	0.78	1.14	1.09	1.31
P. G.	0.81	0.86	0.96	1.15
J. A. J.	0.70	1.07	—	—
J. A. J.	0.72	1.39	0.99	1.54
S. S.	0.63	1.07	—	—
R. J.	0.82	0.93	—	—
C. Z.	—	—	0.64	1.78
C. Z.	—	—	0.59	1.73
A. K.	—	—	0.88	1.48
M. H.	—	—	0.35	1.30
S. D.	—	—	0.77	1.50
J. F.	0.67	0.74	—	—
L. K.	0.47	1.29	—	—
P. V.	—	—	1.06	0.95

DISCUSSION

We have shown that the kinetics of diffusion in the methods introduced by Seligson follow an exponential curve. Furthermore, the rate of ammonia formation due to the action of alkali is a constant for any one sample. This allows a linear extrapolation back to the start of the diffusion and calculation of the amount of ammonia initially present in the system. Measurements on dialyzed plasma and plasma ultrafiltrates indicate that the proteins are the major precursors of the ammonia formed by the action of alkali.

We recommend the following techniques for investigations of blood and plasma ammonia. Arterial blood should be drawn anaerobically into a heparinized syringe and iced immediately. Heparin is the preferred anticoagulant

because it inhibits adenylic deaminase.[26] The time to the start of diffusion should be less than two hours. It seems reasonable that measurements aimed at making correlations with level of consciousness, be made on arterial blood going to the brain rather than on venous samples draining a muscle area, particularly since the venous sample may be markedly influenced by exercise and stasis. Furthermore, Bessman[27] has shown that muscle removes ammonia from blood, the A-V difference increasing with the arterial level. We prefer to use plasma for ammonia measurements—the plasma values are less variable and there is a lower rate of ammonia formation in the diffusion vessel. Since the blood cells give rise to more ammonia than plasma when mixed with alkali, a variation in hematocrit alone could give rise to a considerable variation in ammonia formation from blood. Finally, calculations of the fraction of the total ammonia present as free ammonia and as ammonium ion require measurement of the total plasma ammonia.

Since ammonia is formed in the diffusion bottles it is obvious that the only way one can obtain an accurate measurement of the ammonia initially present is to measure the rate of formation of ammonia and correct for it. However, one can still use a single diffusion time for routine clinical purposes provided the diffusion time is kept short so that the ammonia formed (αt) is small. For example, using the Seligson K_2CO_3 method and diffusion constants from Tables I and II, the diffusion curve for the standards might be $X_1 = Q_1 [1 - 10^{-7.5t}]$. Assuming a rate of ammonia formation for plasma, $\alpha = 1$ μg per hour per milliliter, the diffusion curve for such a plasma will be $X_2 = [Q_2 - 0.087] [1 - 10^{-5t}] + t$. If standards and plasma are diffused for the same time, the

TABLE V. CALCULATED AMMONIA FOUND IN PLASMA AT DIFFERENT DIFFUSION TIMES

Q_2 AMMONIA INITIALLY PRESENT μg/ML.	AMMONIA FOUND AT DIFFUSION TIMES*†		
	t = 10 MIN.	t = 15 MIN.	t = 20 MIN.
0.5	0.55	0.65	0.74
1.0	1.00	1.13	1.23
2.0	1.91	2.08	2.21
4.0	3.61	3.99	4.16

*Calculated for $\alpha = 1$ μg per hour per milliliter, $k_1 = 7.5$ $(hr.)^{-1}$, $k_2 = 5.0$ $(hr.)^{-1}$.
†Ammonia measured by comparing with standards diffused for the same time period.

amounts of ammonia found in the plasma are shown in Table V. Although the errors may be large percentagewise, they are not much larger than the error of the method. However, it must be stressed that these deviations represent a bias, the sign of which depends on the value of Q_2, α, and k_1 and k_2. The random error in a determination using a single short diffusion time is probably larger than the error in our method because the diffusion is stopped while the diffusion process is on the rising portion of the curve instead of the plateau for the standards or the linear portion for the plasma—this introduces an error due to small variations in diffusion time. This is one argument for the use of a slow diffusion process, such as the Seligson K_2CO_3–$KHCO_3$ method, when a single diffusion time must be used.

The discussion so far serves to highlight a drawback in much of the work in this field. We believe that it is the responsibility of each investigator publishing results on ammonia determinations, to run a few complete diffusion curves on standards and plasma and blood samples and to report the average diffusion constants and the rates of ammonia formation in plasma and blood with the method used. Only then will we be able to compare methods and results adequately. To cite a few examples in which the principles outlined in this paper were not followed, Phillips and associates[6] and Traeger and coworkers[11] used the Conway method with whole blood and a 1-hour diffusion. This diffusion time is so long that most of the ammonia found was probably formed in the diffusion vessel. This explains their high normal values. If any correlation exists between blood ammonia and mental status, it might be obscured in such measurements. Zimmerman and associates[12] diffused plasma for 90 minutes at 10° C. There was a 40 to 60 minute interval between the drawing of the blood and the start of the diffusion. They do not state whether the blood was iced or not.

SUMMARY

Studies of diffusion methods for determining blood ammonia have demonstrated:

1. The diffusion of standards follows the exponential curve, $X = Q\ [1 - 10^{-kt}]$.

2. The diffusion curves for blood and plasma are given by $X = \left[Q - \frac{\alpha}{2.3k}\right][1 - 10^{-kt}] + \alpha t$, wherein α is the rate of ammonia formation in the diffusion vessel. For t large, this equation becomes $X = [Q - \frac{\alpha}{2.3k}] + \alpha t$. The determination of a number of points on this linear portion of the diffusion curve and extrapolation to $t = 0$ is the basis for a highly accurate method of measuring ammonia.

3. The proteins in plasma and blood are the major precursors from which ammonia is formed by the action of alkali.

We wish to thank Drs. P. Vanamee and J. W. Poppell for their encouragement and many helpful discussions and Drs. H. T. Randall and C. C. Stock for their support in the carrying out of this work.

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A MICROMETHOD FOR SERUM IRON DETERMINATION

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A MICROMETHOD for the determination of serum iron has been developed following the conditions of the method of Ramsay[1] as later modified[2] to use sodium sulfite as the reducing agent. It has proved useful for studies on small laboratory animals and for surveys among infants and children where only capillary blood was available.

METHODS

Approximately 60 μl of serum in a calibrated Lang-Levy micropipette are placed in a 6 by 50 mm. test tube. To this is added 100 μl of iron-free water, 40 μl of 0.1 M sodium sulfite, and 40 μl of 0.5 per cent 2,2'-dipyridyl in 0.5 M acetic buffer of pH 4.0. Following complete mixing on a high-speed drill the samples are allowed to remain at room temperature for 30 minutes. They are then placed in a boiling water bath for 5 minutes. After about 3 minutes in the water bath, each tube is agitated by hand, permitting more complete separation of the protein precipitate. From 12 to 24 hours later the coagulated proteins are separated by centrifugation and the supernatant is read in a Beckman D.U. spectrophotometer at 510 mμ using the ultramicro cells described by Lowry and Bessey.[3]

Standards consisting of 60 μl of a standard solution of iron made by dissolving iron wire in concentrated hydrochloric acid are run as for serum at concentrations varying from 20 to 500 μg per 100 ml. Blanks containing iron-free water instead of serum are prepared with each set of determinations.

RESULTS

The depth of color developed is proportional to the concentration of iron. There is a constant relationship between concentration and density. This factor has varied less than ± 5 per cent during the last 3 years. A recovery experiment on 14 serum samples containing 20 to 390 μg per 100 ml. of iron was carried out by adding enough of the standard iron solution to the iron-free water to raise the concentration in the serum by 104 μg per 100 ml. The average recovery was 99.0 per cent with a standard error of 1.3 per cent. Assuming that there was no error in the amounts of added iron, the standard deviation between duplicate determinations was 3.4 μg per 100 ml.

In 1956, duplicate analyses of 100 consecutive samples of serum with iron concentrations ranging from 10 to 320 μg per 100 ml. showed a mean difference between duplicates of 10.2 μg per 100 ml. with a standard deviation between duplicate determinations of 10.1 μg per 100 ml. No relation of variation

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between duplicates to the serum iron was found. The difference between duplicates was less than 10 μg per 100 ml. in 59 per cent of the determinations (see Table I). The most recent 100 consecutive duplicate determinations by another technician showed a mean difference of 4.2 μg per 100 ml. with a standard deviation between duplicates of 3.8 μg per 100 ml.

TABLE I. DISTRIBUTION OF DIFFERENCES BETWEEN DUPLICATES IN 100 CONSECUTIVE DETERMINATIONS

DIFFERENCE BETWEEN DUPLICATES (μG/100ML.)	PER CENT OF 100 SAMPLES
0- 9	59
10-19	27
20-29	7
30-39	5
40+	2

DISCUSSION

This micromodification of the modified method of Ramsay has proved to be simple and convenient. The 100 consecutive determinations with the standard deviation between duplicates of 10.1 μg per 100 ml. were done by a person who had just learned the method. The greater precision obtained by her in the recovery experiments and by another experienced worker two years later indicate that considerably greater precision can be achieved with practice. In the last group differences between duplicates never exceeded 14 μg per 100 ml. The distribution in Table I suggests that gross errors occurred in approximately 5 per cent of the determinations and that these could be eliminated by experience.

Since up to 20 samples can be run at the same time with little more effort than that required for a single determination, this method would seem to be applicable to nutrition surveys using capillary blood samples and also to work with small animals. Serum samples can be stored in the frozen state for at least 3 months without appreciable change in serum iron concentration.

This method has been used for 3 years with over 1,000 determinations in the hands of 4 different technicians with consistently reliable results as judged by their correlation with other measures of iron nutrition in a wide variety of hospitalized patients.

SUMMARY

A micromodification of the modified serum iron method of Ramsay has been devised. The standard deviation of variation between duplicates is 3.8 μg per 100 ml. The average recovery of iron added to serum samples was 99.0 ± 1.3 per cent. This method appears suitable for use in nutrition surveys.

The technical assistance of Mrs. Willard Faulkner, Mrs. S. H. Pierce, Jr., and Miss Nancy Rogers is gratefully acknowledged. Mr. Edwin Bridgforth kindly performed the statistical analyses.

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CONTINUOUS ELECTROPHORESIS

Quantitative Fractionation of Serum Proteins

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EXTENSIVE work has been done on the use of filter paper for the separation of serum proteins.[3-11, 13-15] At the present time clinical methods are available for routine electrophoretic serum protein analyses. However, these methods do not provide the researcher with sufficient quantities of these fractions to perform specific biochemical tests. Therefore, this demand for quantity brought about the adaptation of a two phase partition system, continuous flow electrophoresis.

Continuous flow electrophoresis on paper as applied here differs from batch-type anticonvection electrophoresis in that the more slowly migrating components are not required to migrate over a path which has been previously traversed (and perhaps contaminated by adsorption) by the more rapidly migrating components, i.e., albumin tailing.[1] This paper deals with the technical aspects of a specific continuous flow electrophoresis system used by the authors to separate serum into albumin and its globulins in sufficient quantity and purity for biochemical analysis.

MATERIALS AND METHODS

Reagents and Equipment.—

1. Normal serum from fasting subject, 30 ml.

2. Veronal buffer pH 8.6, 0.02 ionic strength, 9 L. Sodium barbital, 1.65 Gm. Barbituric acid, 2.51 Gm. Sodium hydroxide, 0.40 Gm. Dissolve in distilled water and make up to 1 L.

3. Bromphenol blue crystals (for pH work).

4. Acid fuchsin dye, 5 per cent with food coloring. Dissolve 5 Gm. acid fuchsin (acid rubin CI 692) in 100 ml. distilled water. Add approximately 1 Gm. commercial blue food coloring.

5. Polyvinyl pyrollidine (Plasdone C), 30 per cent: 300 Gm. polyvinyl pyrollidine dissolved in 900 ml. of distilled water, with heating. Allow to cool and make up to 1 L.

6. Spinco Model CP continuous flow electrophoresis cell with Constat power source.

7. Test tubes, 15 by 100 mm., for collection of fractions (total of 32).

8. Schleicher & Schuell No. 470 filter paper sheet for upper curtain.

9. Whatman 3 mm. filter paper sheet for lower curtain.

10. Parlodion bags for concentrating collected fractions.[12]

A. Preparing 10 per cent celloidin: (1) Into a 150 ml. brown bottle with a Bakelite lid, place 10 Gm. of Parlodion (Mallinckrodt), 45 ml. of absolute ethyl alcohol, and 45 ml.

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of purified ethyl ether. (2) Shake this mixture vigorously several times a day. In 2 or 3 days the parlodion should be completely dissolved.

B. Molding the celloidin: (1) Into a scrupulously clean tapered 15 ml. centrifuge tube pour 15 ml. of 10 per cent celloidin. (2) Decant the celloidin back into the brown bottle while holding the tube at approximately 70 degrees from the horizontal plane, at the same time turning the tube slowly about its longitudinal axis. This is continued for about 5 minutes. (3) The tube, still inverted, is placed in a rack and allowed to semidry in a vertical position. This requires *10 minutes* (do not dry longer). (4) Fill the tube with water, loosen the celloidin from the lip of the tube with a stick, and gently lift the bag out. (5) Trim the bag 5 cm. from the apex, rinse it several times in distilled water, then place it in distilled water for at least 30 minutes before the testing procedure. The celloidin bag is never allowed to dry, but is kept moist with mild solutions.

C. Testing celloidin bag: (1) Fill the bag with water and insert it into a vacuum chamber (approximately 20 cm. Hg). (2) Any leaks will be apparent by rapid emptying of the celloidin bag.

11. Model R electrophoresis system (Spinco Division, Beckman Instruments Co.).

12. Visking cellulose casing for dialysis of whole serum.

13. Refrigeration unit for cooling of buffer to be circulated through apparatus.

Procedure.—

1. Preparation of serum: Approximately 30 ml. nonhemolyzed serum from a fasting subject is subjected to dialysis against 1 liter of 0.02 ionic strength veronal buffer with 3 changes of buffer. This is done at 5° C. for approximately 20 hours. A few crystals of bromphenol blue are added to the dialyzed serum to produce a faint blue color. Care must be taken to avoid an excess of bromphenol blue. A routine paper strip electrophoretic pattern was run on the serum after dialysis (Table I).

TABLE I. RELATIVE PERCENTAGE DISTRIBUTION OF SERUM PROTEINS PRIOR TO SEPARATION ON THE CP UNIT AS OBTAINED BY ROUTINE PAPER STRIP ELECTROPHORESIS

	PER CENT DISTRIBUTION				
	GAMMA	BETA	ALPHA-2	ALPHA-1	ALBUMIN
Subject serum (after dialysis)	16.5	13.7	7.1	3.3	59.4
Normal	12.0-16.4	9.9-12.9	6.3-9.3	2.6-3.8	60.4-66.4

2. Balancing the system: The refrigerated buffer solution is circulated through the apparatus overnight to bring the electrophoresis unit to a low constant temperature. The following morning the Whatman 3 mm. sheet is thoroughly wetted with buffer and placed in position in the apparatus. The sheet is washed for 30 minutes with circulating buffer, the drain and overflow being discarded. The drain and overflow tubing are then again placed in the buffer reservoir and the curtain is now ready to be balanced.

The flow of buffer from the upper to the lower curtain is regulated by the level of buffer in the upper reservoir which is maintained at a setting of 10 cm.

The flow of buffer from wicks to the lower curtain is regulated to that of the flow from the upper to the lower curtain by placing drops of dye near the top and at the middle on both sides of the lower curtain. The flow of buffer is adjusted until the drops of dye move vertically indicating that both the wick flow and the curtain flow are vertical. The curtain is now balanced.

3. Separation and identification of protein fractions: The dialyzed serum is placed in the 20 mm. diameter vessel (previously washed with buffer) and the vessel placed in position on the instrument. The filter paper sheet is allowed to equilibrate for about 3 hours before current is applied and serum flow started. A constant current of 18 Ma.

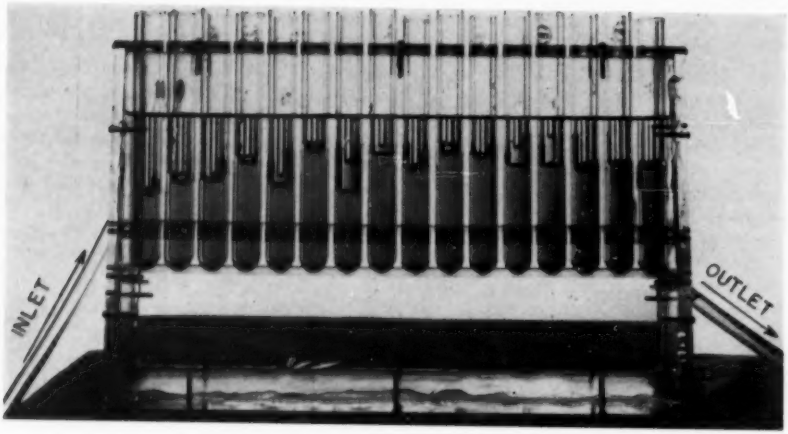

Fig. 1.—Refrigerated collection rack.

Fig. 2.—Lower curtain stained with bromphenol blue upon completion of separation.

(650 volts) is applied by the Constat power source. The sample flow rate mechanism is actuated. The feed rate duodial setting of 1.0 (0.15 ml. per hour) was found to be the most desirable rate of serum flow to the curtain.

This is allowed to run overnight in this manner and collection started next morning after it was observed that the bromphenol blue was visible at tab 29 indicating good resolution of the albumin. (Good resolution of albumin is usually indicative of good resolution of the globulins if the curtain has been properly aligned.)

A special enclosed rack has been designed by the authors to hold the collection tubes and make use of the buffer overflow as a refrigerant for the collected fractions (Fig. 1).

Collections of the various fractions were made over a 3-day period. Tubes containing the collections were placed in the refrigerator until ready for further analysis.

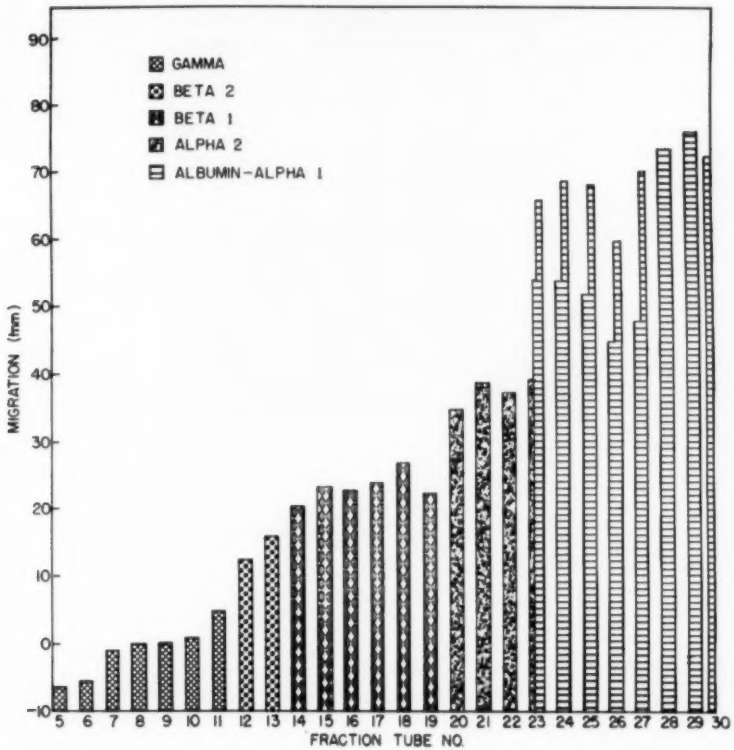

Fig. 3.—Identification of protein fractions by preferential mobilities.

When fractionation of the serum was completed color photographs were taken to attempt to show the location of the bromphenol blue on the curtain and in the collection tubes; also to show the variability of color in the specific protein fractions.

The lower curtain upon completion of the run was dried and stained with bromphenol blue, 0.1 per cent alcoholic stain to indicate the approximate location of the protein fractions (Fig. 2).

The contents from each collection tube were concentrated to approximately one-third the original volume (Fig 4). The volumes prior to and after concentration are recorded. Total proteins were performed on the concentrated protein fractions.

Routine paper strip electrophoretic patterns were performed on each concentrated fraction,[3] the identification of each fraction being indicated by its mobility.

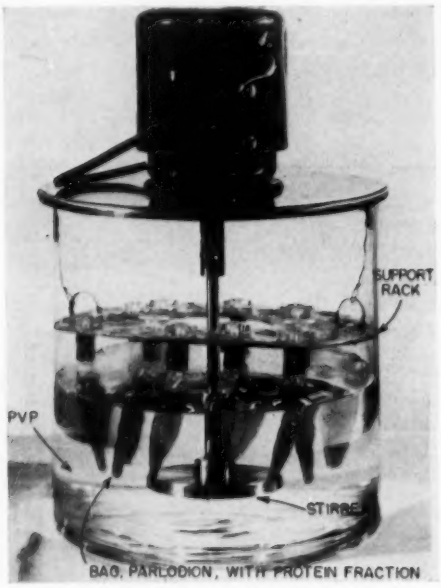

Fig. 4.—Apparatus for concentration of continuous flow fractions.

RESULTS

In order to obtain an absolute value relationship between each fraction collected, the following formula is applied:

$$\frac{\text{TP (mg./ml.)} \times \text{ml. (concentrate)}}{\text{ml. (original volume)}} \times 10 = \text{mg./10 ml.}$$

The resultant values are shown in Table II.

The identification of the albumin and globulin fractions is indicated by preferential mobilities as shown in Fig. 3.

DISCUSSION

Although continuous electrophoresis has been described, in part, elsewhere[1, 2] the authors present here a complete procedure for the preparation of specific serum fractions for further chemical analyses. Certain techniques have been incorporated to aid in obtaining more quantitative results.

A detailed structural description of the apparatus in use can be found in Chapter 18 of Reference 1.

In the preparation of the serum a small amount of bromphenol blue is added to aid in location of albumin on the lower curtain. When serum to which a minute quantity of bromphenol blue has been added is subjected to an electric field, the bromphenol blue, having an affinity for adsorption onto the albumin, migrates with the albumin.

TABLE II. DESCRIPTION OF COLLECTION OF TUBE CONTENTS

TUBE NO.	VOLUME (ML.)		TOTAL PROTEIN		DESCRIPTION OF CONCENTRATED SAMPLE
	ORIGINAL	CONCENTRATED	UNCORRECTED (GM. %)	CORRECTED (MG./10 ML.)	
3	6.7	1.2	1.1	19.7	White precipitate
4	6.2	1.3	1.9	39.9	White precipitate
5	5.0	1.3	1.3	33.8	Clear
6	6.5	1.0	2.1	32.3	Clear
7	6.0	1.5	0.830	20.7	White precipitate
8	7.4	1.1	1.1	16.3	Clear
9	5.3	1.4	0.554	14.6	Clear
10	6.6	1.3	0.554	10.9	Clear
11	4.2	1.4	0.400	13.3	Clear
12	6.7	1.6	0.276	6.6	Clear
13	6.5	1.1	2.1	35.5	Clear, yellow-orange
14	4.9	1.4	0.982	28.1	Turbid, yellow-orange
15	5.6	1.3	1.72	40.0	Clear, yellow-orange
16	5.2	1.7	0.400	13.1	Turbid precipitate, yellow-orange
17	4.8	1.3	0.675	18.3	Clear, yellow-orange
18	6.1	1.0	0.554	9.1	Clear, yellow
19	5.5	1.9	0.400	13.8	Clear, yellow
20	6.8	1.0	1.1	16.2	Turbid
21	5.9	1.3	0.982	21.6	Turbid
22	7.2	1.7	0.554	13.1	Slightly turbid
23	6.0	1.3	1.1	23.8	Very slightly turbid
24	7.2	1.2	2.82	47.0	Clear, blue-green
25	7.3	1.6	4.4	96.4	Clear, blue-green
26	8.0	1.8	4.4	99.0	Clear, green
27	7.9	1.7	4.95	106.5	Clear, blue-green
28	9.3	1.2	3.8	Lost	Clear, blue-green
29	10.2	1.5	2.1	30.9	Clear, blue
30	14.3	1.3	0.123	Lost	Clear, blue

However, if the adsorption quality of the albumin is exceeded by the bromphenol blue, it has been found that the dye migrates at a more rapid rate just ahead of the albumin.[2]

Circulation of the buffer at approximately 1° C. affords a sufficiently refrigerated closed system to prevent diffusion due to heat convection caused by the electrical potential across the lower curtain.

Fluctuations in the system will be observed by any change in the position of the light blue streak caused by the bromphenol blue adsorbed onto the albumin. In order to obtain pure fractions it is imperative that the bromphenol blue streak does not change its position on the lower curtain. The following factors must be held constant throughout the entire separation.

1. Curtain flow
2. Wick flow
3. Current across lower curtain

4. Serum feed to lower curtain

5. Temperature of circulating buffer

Special care must be taken to balance the system to avoid any flow from the wicks into the curtain since electrode products, if allowed to enter the lower curtain, will form more strongly basic and acidic regions relative to the pH of the buffer from the upper to the lower curtain.

Certain factors should be considered in the process of concentrating the resultant fractionated serum. If the degree of concentration is too great there is the danger of precipitating specific proteins by forming a hypertonic salt solution. Although this precipitation is reversible in most instances, certain lipid containing proteins are irreparably damaged. This factor must be considered when using salt concentrations greater than 0.02 ionic strength or lower curtains with a faster flow rate.

Another advantage of using electrolytes of low ionic strength is that they allow higher intensity electrical fields to be applied with relatively low current flow.

Although the alpha-1 globulin was not quantitatively separated from the albumin on this first separation, we feel that by combining tubes 23 to 30, refractionating at a higher milliamperage, and repositioning of the point of inoculation, the alpha-1 globulin could be separated from the albumin. However, the diagnostic significance of an altered alpha-1 globulin is of questionable value and therefore further attempts to separate this fraction from the albumin were not performed.

Although we have pointed out in this paper the first separation of serum proteins by continuous electrophoresis, work has been done by this laboratory for further fractionation studies. Therefore, one should keep in mind that once, for example, the gamma globulin is separated in tubes 5 through 11 these fractions may be pooled, concentrated, and further fractionated on another curtain using a different electrical field strength.

Tubes 3 and 4 contain protein considered to be part of the gamma fraction (see Table II). The migration of protein this close to the extremities of the curtain should be avoided whenever possible. Although not shown here, tubes 1 and 2 showed an absence of protein as indicated by biuret reaction.

SUMMARY

The problems involved in obtaining quantitative reproducibility in the isolation of specific protein fractions in quantity are many. The technical difficulties in the separation and identification of specific serum proteins have been resolved by the authors incorporating a commercially available continuous flow electrophoresis apparatus.

The approach to the solution of technical difficulties involved in a method by which specific serum proteins may be obtained in sufficient volume for chemical evaluation is thoroughly discussed.

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A COMPARISON OF TWO SAMPLING METHODS FOR THE ISOLATION OF ADENOVIRUS

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AN IMPORTANT phase of an epidemiologic study of infectious diseases is the isolation and identification of etiologic agents. Successful isolation of a communicable disease agent is interdependent on a variety of factors, such as the individuals sampled, time after onset of illness, source of specimen, method of sampling, storage, and culturing of sample material. Of these variables, the method of sampling was selected for analysis.

In the study of the relationship of the adenoviruses to acute respiratory disease essentially 2 sampling methods are used for the isolation of this agent. Hilleman and associates[1-3] indicate preference for the throat washing mode whereas investigators of the United States Public Health Service have used the throat swab technique.[4-6] In one instance[6] both throat swab and throat washing procedures were employed. In adenovirus studies of children, Bell[7] prefers use of the throat swab since many of these subjects are unable to gargle. Hence, an experiment was initiated in the Recruit Training Command, Great Lakes, Illinois, to compare the throat washing and throat swab techniques.

The results obtained show no significant difference in numbers of adenoviruses isolated by either method and that the total number of recruits yielding virus (by combining the results of both methods) exceeded the number of isolations by either method alone.

MATERIALS AND METHODS

Sampling Methods.—Specimens were obtained from Navy recruits reporting to a dispensary with clinical symptoms indicative of an acute respiratory infection. Throat swabs and throat washings were obtained simultaneously from the same individual. The maintenance medium of Friedman[8] was used as diluent for both swabs and washings. Sterile diluent was distributed as 2 ml. volumes into 15 by 40 mm. screw-cap vials and for throat swabs as 20 ml. volumes in 25 by 150 mm. tubes for throat washings. Penicillin (200 units per milliliter) and streptomycin (200 μg per milliliter) were added to the small vial in which swabs were to be placed. Antibiotics were not added to the throat gargles to avoid possible reactions in penicillin-sensitive individuals. The tubes and vials were stored at −20° C. and were warmed at 37° C. before use in order to liquefy the gelatin component of the medium.

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The throat swab specimen was obtained by rubbing a sterile, cotton-tipped applicator stick against the lateral and posterior regions of the pharynx. The swab portion was then inserted into the small vial, and the stick broken off to a length convenient for securing the cap with the swab portion retained in the vial.

After collecting the swab sample the throat washing was obtained by having the patient gargle with 20 ml. of the sampling diluent for 15 seconds. The expectorate was collected, and a 2 ml. aliquot of this material was frozen in a −60° C. mechanical freezer.

Tissue Culture Methods.—The method used for adenovirus isolation and identification has been described[3, 5] but was modified as follows. Single HeLa cell test tube cultures were inoculated with 0.2 ml. of throat swab diluent but with only 0.1 ml. of the throat washing material, since the latter was found to be considerably more toxic for the HeLa cells. Adenovirus isolates were typed by the neutralization test with the following modification. One-tenth milliliter of type-specific advenovirus immune rabbit sera and 0.1 ml. of virus material were mixed directly in washed HeLa cell tubes containing 0.8 ml. of maintenance media. This was in lieu of a separate preincubation of virus and antiserum. This time-saving modification was used in view of adenovirus growth curve studies of Ginsberg[9] and similar work of Lieberman and Friedman,[10] which revealed that adenovirus was absorbed to HeLa cells at a relatively slow rate.

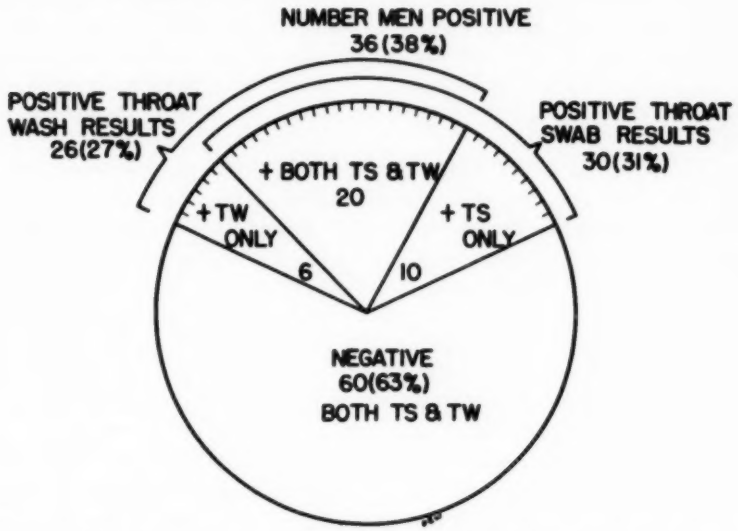

Fig. 1.—Adenovirus isolations from throat swabs (TS) and throat washings (TW) simultaneously obtained from 96 hospitalized Navy recruits.

RESULTS

The results of the virus isolation comparison study are shown in Table I. Of 100 individuals sampled, 4 specimens were contaminated in either one sampling method or the other and were not included in the analysis. From the remaining 96 men, 26 adenoviruses were isolated by the throat washing method as compared to 30 isolations from throat swabs. Fig. 1 shows total number of men (36) from whom adenovirus was recovered as determined by combining the results of both methods.

Approximately 97 per cent of all adenoviruses isolated were Type 4 with only one Type 3 which was recovered from the same man by both methods. In addition, the data show that both methods agreed in 80 instances of positive and negative findings. However, there is nonconcurrence in 16 observations where a virus was isolated from one method and not the other.

There was no significant difference in number of virus isolates obtained by either method, as determined by the chi-square test.

TABLE I. NUMBER OF MEN POSITIVE FOR ADENOVIRUS ISOLATION*

SAMPLING METHOD	POSITIVE	PER CENT POSITIVE
Throat washing	26	27
Throat swab	30	31
Combined	36	38

*A total of 96 recruits were in the sampling group.

DISCUSSION AND SUMMARY

The present study indicates that there was no significant difference in ability of either sampling method to isolate adenovirus Type 4. However, combining the results of both methods increases the total number of men found to be positive. This might be similar to obtaining 2 samples using the same method on the same individual thus increasing the number of samples to be tested.

The mechanical action of the throat gargle differs considerably from the throat swab. The gargle covers a greater surface area and is able to permeate crevices inaccessible to the throat swab. However, the traumatic effect of swabbing may cause a greater release of intracellular virus than throat washing. The greater volumes of throat washing fluid that must be used cause a proportionate increase in virus dilution. Moreover, the throat washing fluid is considerably more toxic for HeLa cells than the throat swab material, making it necessary to use smaller volumes of throat washing for primary isolations. Despite these variations, the results in terms of virus isolation appear to show no significant differences in the two methods.

The practical advantages of the throat swab method are (1) simplicity of its preparation and its small size for carriage and storing, (2) ease of manipulation in both sampling and in tissue culture inoculation, and (3) greater acceptability to patients.

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IMPROVEMENTS IN METHODS FOR MEASURING HYDROXYPROLINE

Application to Human Urine

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SINCE hydroxyproline is uniquely present in a high concentration in collagen, the determination of this amino acid serves as a method for estimating the collagen content of animal tissues. The measurement of hydroxyproline in blood and urine may also be an index of over-all collagen metabolism. The methods most commonly employed in the colorimetric assay of hydroxyproline are those of Neuman and Logan[1] and Wiss.[2] However, the former method lacks specificity while the latter, although specific, gives an unstable chromophore. We wish to report certain modifications in both methods which have eliminated these problems. The application of one of these methods to the measurement of urinary hydroxyproline in a variety of clinical conditions is also presented.

MATERIALS AND METHODS

Modification of the Neuman and Logan Method.—The modification is based on the findings reported by Radhakrishnan and Meister[3] which showed that treatment of hydroxyproline with $CuSO_4$ and H_2O_2 in an alkaline solution as in the Neuman and Logan method resulted in the formation of Δ^1-pyrroline-4-carboxylic acid. Upon acidification this compound was converted to pyrrole-2-carboxylic acid which condenses with *p*-dimethylaminobenzaldehyde to give the colored complex. The present procedure involves extraction of pyrrole-2-carboxylic acid with ether to separate it from the degradation products formed from such compounds as tyrosine and tryptophan, thus, conferring added specificity to the method.

Two milliliters of a sample containing up to 50 μg of hydroxyproline is placed in a 50 ml. shaking tube. One milliliter each of 2.5 N NaOH, 0.01 M $CuSO_4$ and 6 per cent H_2O_2 is added, and the mixture is left at room temperature for 5 minutes, after which the excess peroxide is removed by heating at 80° C. for 5 minutes. The tube is then cooled, 0.5 ml. of 6 N H_2SO_4 is added and heated in a 60° C. bath for 10 minutes. The solution is cooled and extracted with 15 ml. of ether. Ten milliliters of ether is then transferred to another shaking tube containing 3 ml. of 0.1 N NaOH. After the ether is extracted with the base it is removed by suction and discarded. To 2 ml. of the base is added 3 ml. of 2:1 H_2SO_4 (3 N) and *p*-dimethylaminobenzaldehyde (5 per cent solution in n-propanol), and the mixture is heated at 70° C. for 16 minutes. The solution is cooled and read at 540 mμ in a 20 by 100 mm. cuvette using the Spectronic 20 spectrophotometer.

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Modification of Wiss Method.—The rapid formation and fading of the chromophore described by Wiss in his method is due to the concentrated hydrochloric acid used in the procedure. It was found that under controlled heating in a weaker acid, a stable chromophore developed.

Five milliliters of sample containing 2 to 10 μg of hydroxyproline is placed in a 50 ml. round bottom flask, and 0.5 ml. each of chilled 10 per cent Na_2CO_3 and hypochlorite (7.1 per cent chlorine solution in 8 per cent NaOH) is added. After the mixture stands for 3 minutes in an ice bath, 1.5 ml. of glutamic acid solution (10 per cent glutamic acid, neutralized) is added and the mixture is heated until 5 to 6 ml. is distilled. The distillate is made up to 7 ml. to which are added 2 ml. of *p*-dimethylaminobenzaldehyde (2.5 per cent solution in ethanol) and 1 ml. of 2 N H_2SO_4. The mixture is heated for 20 minutes at 60° C. The solution is cooled and read at 550 mμ in a 25 by 100 mm. cuvette using the Coleman Junior spectrophotometer.

Total Hydroxyproline in Urine.—To 2 ml. of urine 8 ml. of saturated $Ba(OH)_2$ solution is added and the mixture is autoclaved overnight at a pressure of 18 to 20 pounds. The cooled solution is neutralized with 2 N H_2SO_4, centrifuged, and the supernatant fluid is adjusted to 10 ml. Aliquots of 1.5 to 5 ml. are assayed by the modified method of Wiss.

Urinary Peptide.—This was determined by the method of Balikov and Castello.[4]

Clinical Material.—Twenty-four hour collections of urine were obtained on 76 individuals. All but 4 were adults ranging in age from 17 to 77 years. Twelve were normal volunteers; 10 were diagnosed as having Marfan's syndrome; 6 the Ehlers-Danlos syndrome; 4 the carcinoid syndrome; and 1 case each of argyria, multiple myeloma, and nonpulmonary hypertrophic osteoarthropathy. Thirty-eight had miscellaneous diseases as indicated in Table II.

TABLE I. COMPARISON OF THE DATA OBTAINED BY THE ORIGINAL AND MODIFIED METHODS OF NEUMAN AND LOGAN AND BY THE MODIFIED METHOD OF WISS

MATERIAL	ORIGINAL NEUMAN AND LOGAN HYDROXYPROLINE EQUIVALENCE	MODIFIED NEUMAN AND LOGAN HYDROXYPROLINE EQUIVALENCE	MODIFIED WISS HYDROXYPROLINE EQUIVALENCE
Difco Bacto-tryptone, 20 mg.	10.9 μg	3.8 μg	2.1 μg
Tyrosine, 6 μM	15.5 μg	1.1 μg	0.0
Basic hydrolysate of urine from patient with Marfan's syndrome	114.8 mg.	101.6 mg.	101.5 mg.

RESULTS

Specificity of the Modified Method of Neuman and Logan.—As shown in Table I the direct application of the Neuman and Logan method to a solution containing either 20 mg. of a pancreatic casein digest (Bacto-tryptone) or 6 μM of tyrosine resulted in the formation of colored products. Extraneous color formation could be reduced considerably by interposing the ether extraction step described under Methods. The reliability of the modified method was further demonstrated when it was applied to urine and the result compared with that obtained with the more specific Wiss method. With some urines the original method gave values higher than those obtained by the modified method, the values obtained by the latter method agreeing satisfactorily with those of

the Wiss method. In Fig. 1, the relationship between optical density and concentration of hydroxyproline is shown as determined by our modified procedure.

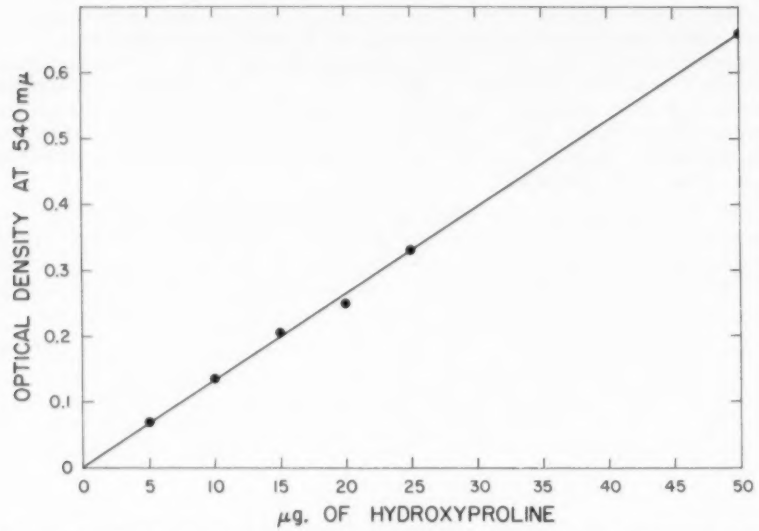
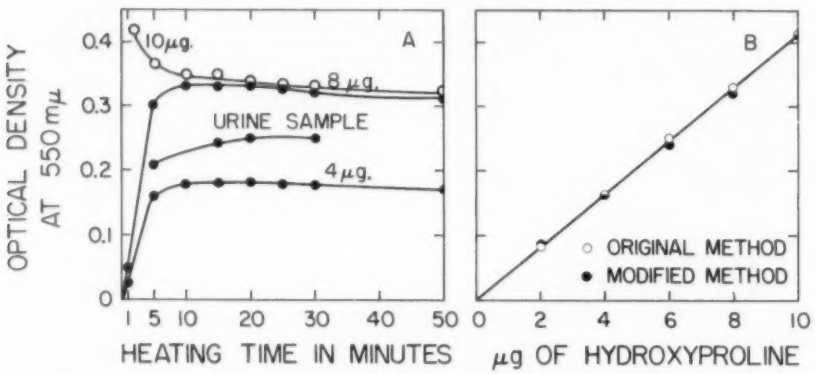


Fig. 1.—Standard curve for hydroxyproline obtained by the modified Neuman and Logan method.



A. *B.*

Fig. 2.—*A*, Development of color at 60° C. in the modified Wiss method in relationship to time and comparison with the original method. *B*, Standard curve for hydroxyproline by the original and modified Wiss method.

Stability of the Modified Method of Wiss.—As shown in Fig. 2, *A*, when 1 ml. of 2 N H_2SO_4 was used instead of concentrated HCl (22.5 per cent) and color formation was allowed to take place in a water bath at 60° C., maximum color was attained in 10 to 20 minutes with standard hydroxyproline

solutions. With urine the color formation reached a maximum value in 20 minutes. Thus, heating at 60° C. for 20 minutes was chosen as the standard procedure. When the solutions were cooled, the intensity of the color at room temperature was constant for 1 hour, only 4 per cent being lost after 1½ hours. Fig. 2, *B*, shows that the standard curve obtained by the modified procedure is identical with that obtained by the original method of Wiss.

Use of Basic Hydrolysis.—For the analysis of hydroxyproline in urine, Ziff and associates[5] used HCl for hydrolysis which is the common procedure used in hydrolyzing proteins and peptides. Since the preparation and subsequent handling of basic hydrolysates are considerably simpler, $Ba(OH)_2$ was used throughout this work. When analyses for hydroxyproline were conducted on two samples of urine, hydrolyzed as described under the section on Methods and compared with HCl hydrolysates, the results were in reasonable agreement. Thus, basic hydrolysates gave 24-hour excretion values of 43.6 mg. and 36.0 mg., while acid hydrolysates gave 42.1 mg. and 34.4 mg., respectively. When recovery studies were conducted by adding various amounts of gelatin containing 4 to 66 μg of hydroxyproline to urine samples, an average of 96 per cent recovery of the added hydroxyline was obtained after basic hydrolysis of the mixture.

TABLE II. DAILY EXCRETION OF TOTAL HYDROXYPROLINE IN VARIOUS GROUPS

GROUP	NUMBER	SEX	AGE	HYDROXYPROLINE (MG./24 HR.) TOTAL MEDIAN	TOTAL RANGE	FREE MEDIAN	FREE RANGE
Normal adults	12	6M, 6F	18-25	25.1	14.0- 38.7	0.65	0.19-2.16
Miscellaneous diseases*	37	18M, 19F	20-77	22.9	11.8- 42.5	0.89	0.26-3.26
Marfan's syndrome	10	8M, 2F	17-31	61.6	16.0-106.0	2.9	0.55-9.89
Carcinoid syndrome	4	3M, 1F	50-69	34.2	16.4- 42.5	1.1	0.37-3.16
Ehlers-Danlos syndrome	6	4M, 2F	24-62	24.9	14.7- 33.9	2.0	0.50-4.7
Argyria	1	F	36	80.1	—	5.6	—
Multiple myeloma	1	F	64	51.2	—	5.0	—
Nonpulmonary hypertrophic osteoarthropathy	1	M	27	119.9	—	5.1	—
Osteogenesis imperfecta	4	4M	6	31.9	14.1- 41.0	0.36	0.19-0.44

*Diseases included are rheumatoid arthritis, bronchial asthma, arteriosclerotic cardiovascular disease, essential hypertension, ulcerative colitis, regional ileitis, Hodgkin's disease, pulmonary emphysema, scleroderma, hereditary cerebellar ataxia, aneurysm of the aorta (cause unknown), and exogenous obesity. Two patients in the first trimester of pregnancy are also included.

Excretion of Total Hydroxyproline in Various Diseases.—As shown in Table II, the median daily excretion of total hydroxyproline in 12 normal adults was 25.1 mg., the range being 14.0 to 38.7 mg. The free hydroxyproline comprised on the average 2.4 per cent of the total. In 37 adults with miscellaneous diseases, the median excretion of total hydroxyproline was 22.9 mg., the range being 11.8 to 42.5 mg. A definitely higher median value for the total hydroxyproline was obtained in 10 patients with Marfan's syndrome with free hydroxyproline comprising a slightly larger per cent of the total. As shown in Fig. 3, 8 out of 10 of these patients showed daily excretion values higher than the median value in the normals. When equivalent aliquots of hydrolyzed 24-hour urine samples of 2 normal subjects and 2 patients with

Marfan's syndrome were analyzed by paper chromatography and developed for hydroxyproline by the method of Pasieka and Morgan,[6] much brighter spots for hydroxyproline were obtained with the samples from Marfan's syndrome as expected on the basis of colorimetric assays. The urinary peptide values in 8 patients with Marfan's syndrome and 10 normal subjects were identical, the median being 2.5 Gm. per 24 hours; the range for the former group was 2.0 to 3.3 and for the latter, 1.2 to 5.9. The group with Ehlers-Danlos syndrome showed a normal median value for the total hydroxyproline although the free hydroxyproline value appeared to be slightly higher than in the normal subjects. The total hydroxyproline values in the patients with argyria, multiple myeloma, and nonpulmonary hypertrophic osteoarthropathy were higher than the highest value among the normals. The median value obtained in 4 cases of osteogenesis imperfecta among children was 31.9 Gm.

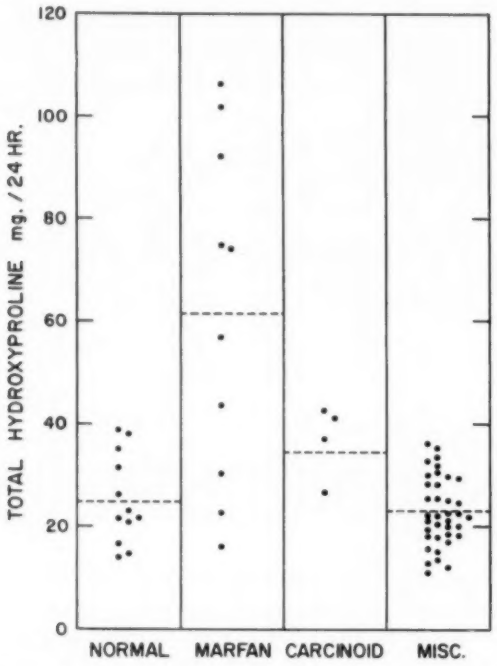


Fig. 3.—Distribution of 24-hour excretion values of total hydroxyproline in various groups.

DISCUSSION

The method of Neuman and Logan is a satisfactory method for establishing the purity of an isolated sample of collagen. However, the method as described should not be applied to assay of hydroxyproline in crude biologic

extracts since other compounds can give rise to similar chromophores. Furthermore, even the modified method of Neuman and Logan cannot be applied to basic hydrolysates of crude biologic extracts since mucoproteins have been shown to give rise to pyrrole-2-carboxylic acid after alkaline treatment.[7] On the other hand, the method of Wiss is very specific for hydroxyproline and does not give rise to color even with pyrrole-2-carboxylic acid. The major difficulty in this method, the instability of the color, has been remedied by the present modification.

The median value for the total hydroxyproline excretion in normal subjects (25.1 mg.) is comparable to that reported by Ziff and associates (21.8 mg.). The agreement between the two groups is really excellent because, first, hydroxyproline is labile in acid. We have found that autoclaving urine with hydrochloric acid beyond 3 hours results in a precipitous fall in the amount of hydroxyproline recovered. Since the acid is concentrated during its removal by evaporation, some destruction of hydroxyproline is unavoidable. Second, our patients were fed an unrestricted diet as contrasted to the patients handled by Ziff's group.

To our knowledge the high median value for total hydroxyproline excretion by patients with Marfan's syndrome represents the first report of an abnormality of hydroxyproline metabolism in patients with a collagenous disease. Ziff reported no significant differences between controls and patients with a variety of collagen diseases. The increase in total hydoxyproline cannot be ascribed to an over-all increase in total amino acid excretion in these patients since the peptide values of these patients were within normal range. In contrast to this, the high excretion of hydroxyproline by the nonpulmonary hypertrophic osteoarthropathy patient was found to be accompanied by a several fold increase in peptide excretion (15 Gm. per day). The reason for this increased excretion of hydroxyproline by patients with Marfan's syndrome is the subject of present investigations in this laboratory.

The significance of the data presented on argyria and multiple myeloma is not known since only 1 case of each was studied. The increase observed in multiple myeloma could be due to the general increase in excreted peptides derived from tissue breakdown. Patients with carcinoid syndrome were included in this study because of certain alterations in their connective tissues. However, no significant abnormality in hydroxyproline excretion was found.

The median value of 39.1 mg. for the daily excretion of hydroxyproline in patients with osteogenesis imperfecta is interesting in that the median for normal children has been reported to be 63.7 mg.[5]

SUMMARY

1. The methods of Neuman and Logan[1] and Wiss[2] have been modified to give, respectively, an added specificity and stability in the chromophore. The simpler basic hydrolysis as compared with acid hydrolysis combined with the modified method of Wiss has been found to be a satisfactory method for assaying urinary hydroxyproline.

2. The method has been applied to normal urine and to urine of patients with a variety of clinical conditions. Patients with Marfan's syndrome were found to excrete larger amounts of hydroxyproline per day than normal controls.

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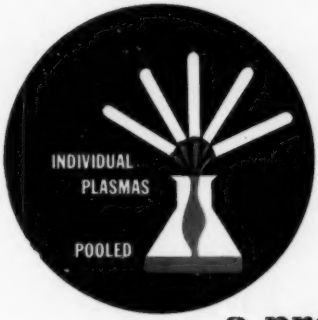

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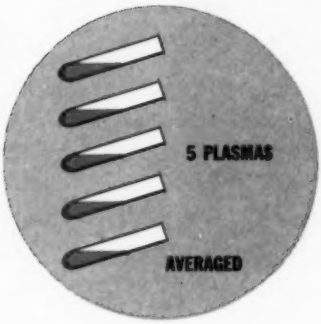

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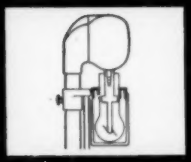

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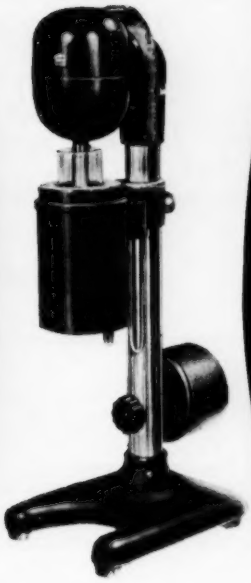

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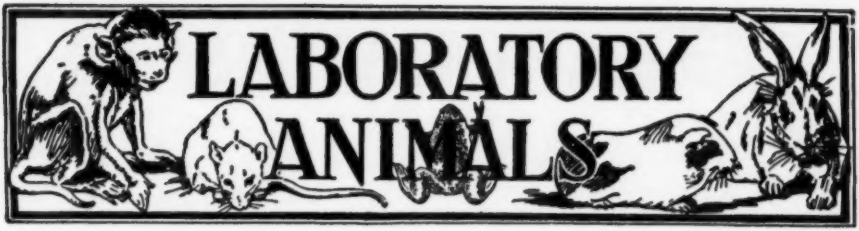

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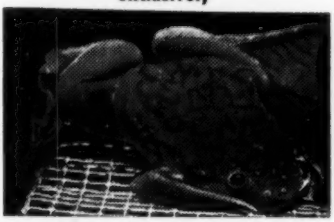

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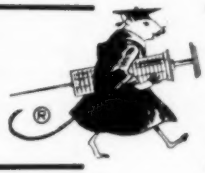

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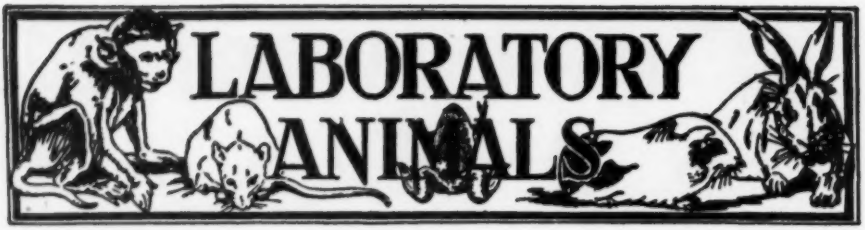

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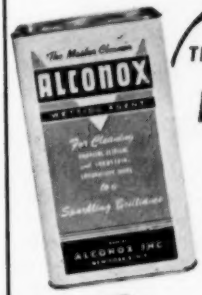

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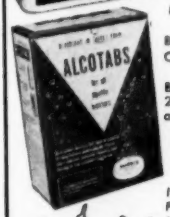

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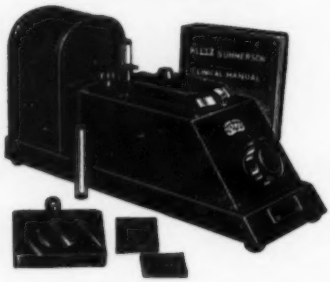

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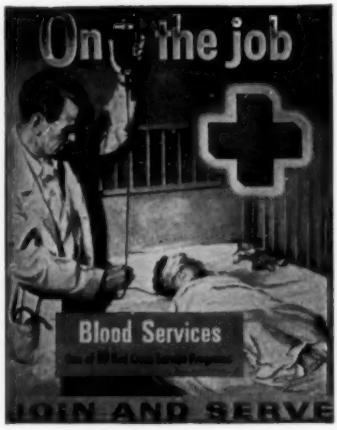

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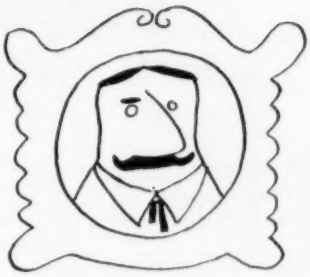

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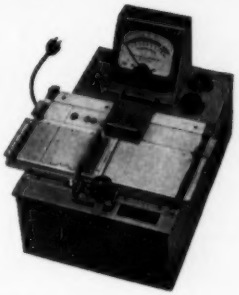

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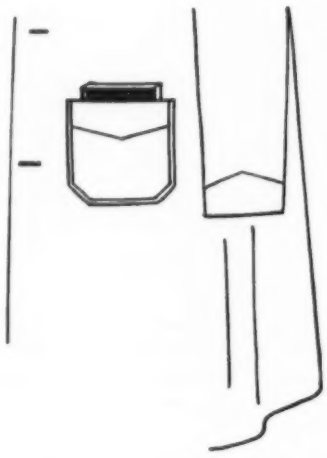

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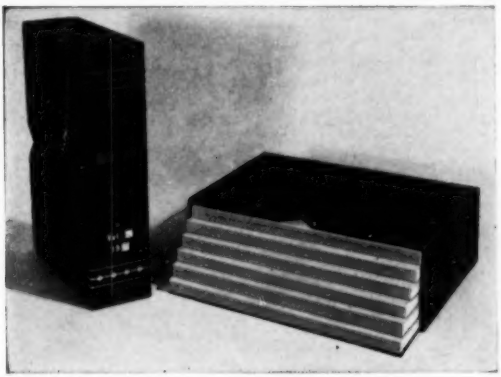

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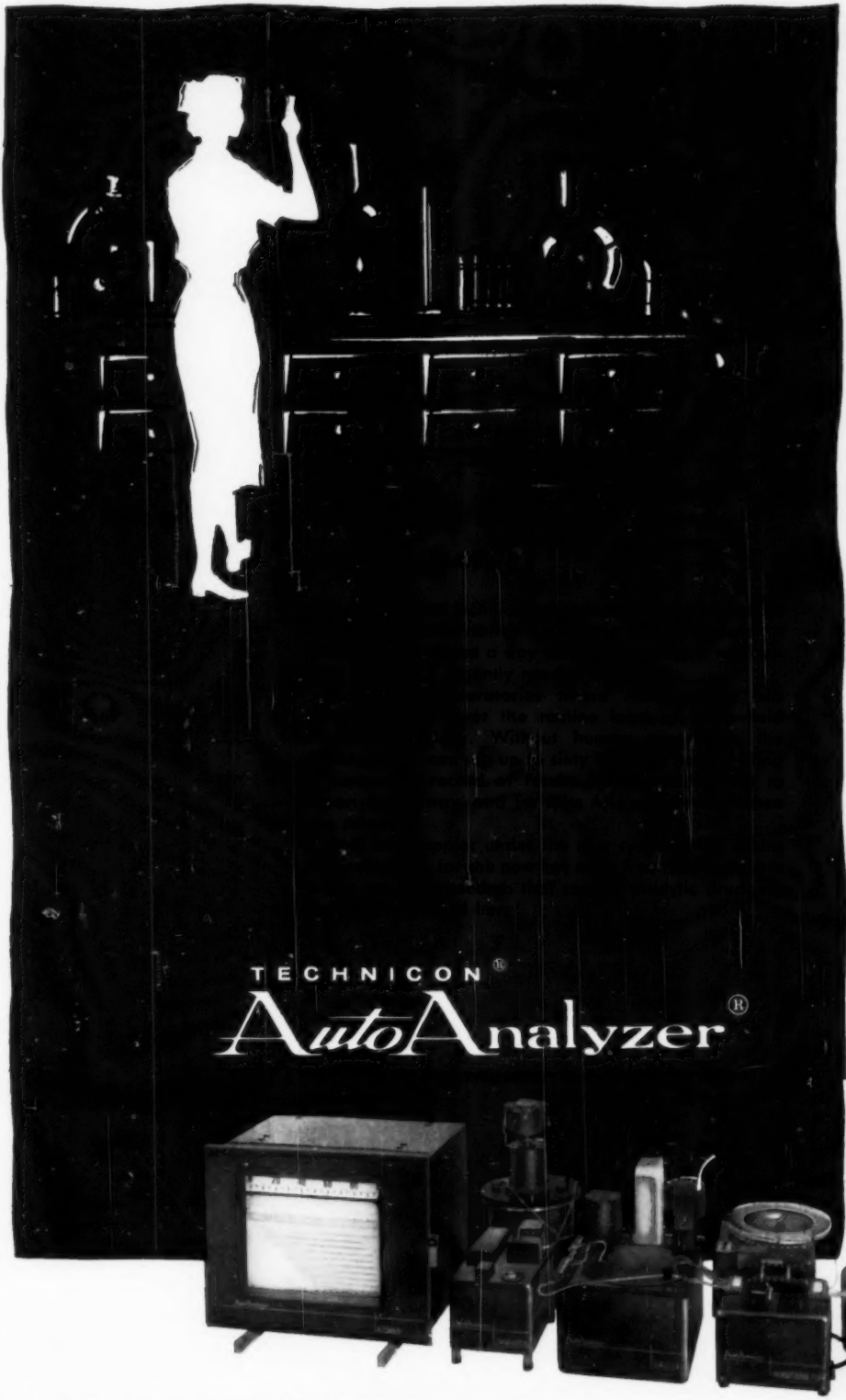

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Source: (1) Diabetes Program Guide, U.S. Department of Health, Education, and Welfare, Chronic Disease Program, 1957, p. 18. (2) Packer, H., and Ackerman, R. F.: Diabetes 7:312, 1958. (3) Ham, T. H.: A Syllabus of Laboratory Examinations in Clinical Diagnosis, Cambridge, Harvard University Press, 1950, p. 18.

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